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TITLE: "Inflammation Modulatory Protein TSG-6 for Chemical Injuries to the Cornea

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14. ABSTRACT  During this study period we established the time sequence of inflammation, and the cellular and molecular changes which occur after exposure of rat corneas to different concentrations of alkali. These findings set the conditions for testing the effectiveness of both topically applied and intraocular TSG-6, an anti-inflammatory protein, in the promotion of corneal healing and restoration after injury. We found that TSG-6 applied in this manner did not provide a statistically significant benefit to the cornea after alkali injury as evaluated by corneal clarity and by biochemical markers of inflammation. Experiments to test the effectiveness of intravenous TSG-6 are in the preliminary stages.				
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## **1. INTRODUCTION:**

This project aims to study the treatment of chemical injury of the cornea with a natural anti-inflammatory protein, TSG-6, which has a novel mechanism of action. Chemical injuries of the eye are difficult to treat, and may lead to severe debilitation or blindness with few patient treatment options. The wartime threat for chemical injury to the eye is evidenced by the thousands of soldiers injured with mustard gas in the Iran-Iraq war. The mechanism of injury after chemical ocular injury includes inflammation secondary to trauma, and may be alleviated with anti-inflammatory agents like TSG-6. TSG-6 works by inhibiting inflammation at the earliest stage, and is effective in the treatment of mild chemical injuries caused by alcohol and mechanical scraping in a mouse model. This study is designed to determine if TSG-6 will be effective with more severe chemical alkali injuries of the cornea, and which would more closely mimic injuries from mustard gas or other severe caustic agents. We will treat rat corneas injured with different concentrations of alkali using topical, anterior chamber, and IV TSG-6 to determine the efficacy and time course of treatment as evaluated by clinical improvement and corneal clearing, and biochemical markers of inflammation.

## **2. KEYWORDS:**

Cornea, alkali injury, eye trauma, chemical injury, anti-inflammatory protein, stem cells, TSG-6, regeneration

## **3. ACCOMPLISHMENTS:**

### **a. Project Goals**

The specific goals of the project for the first year of the project as pertains to the SOW included:

- 1) Specific Aim 1 Subtask 1: Obtain ACURO approval for study initiation
- 2) Specific Aim 1 Subtask 2: Determine the acute phase timing and patterns of inflammatory cytokines and chemokines in response to the severity of alkali injury for 0.1N, 0.5N, and 1N injury to the rat cornea

- 3) Specific Aim 1 Subtask 3: Determine the timing and patterns of cellular changes in response to the severity of alkali injury (histopathology)
- 4) Specific Aim 2 Subtask 1: Determine the efficacy for topical and intraocular TSG-6 after corneal alkali injury to the rat cornea for varying concentrations of alkali

### **3. ACCOMPLISHMENTS, ctd**

#### **b. Goals Achieved**

1) Specific Aim 1 Subtask 1: ACURO approval 24 Sept 2014

2) Specific Aim 1 Subtask 2: Determine the time course of injury for controls, and 0.1N, 0.5N, and 1N injury levels, completed by 1/31/15. Initial experiments at the 0.1N injury level resulted in an injury that was insufficient to distinguish a possible treatment benefit of TSG-6, and an amended SOW was submitted and approved in order to concentrate on the 0.5N and 1N injury levels.

Rat corneas were exposed to varying concentrations of NaOH for 30 seconds using filter paper soaked with NaOH, and then the corneas were thoroughly rinsed with 40cc of BSS. The corneas were photographed for clinical scoring at varying time points, and the corneas were collected for cytokine PCR assay for RNA at varying intervals after injury at 2 hr, 4 hr, and 1,2,3 and 5 days for a total of 6 time points.

The peak of inflammation for the 0.5 N and 1N injury levels from chemokine studies indicates that the peak of inflammation occurs by day 1, and then begins to subside. The inflammatory cytokine peaks at day 1 for IL1B and IL6 are shown to be dependent on level of alkali injury with insignificant levels at 0.1N, which increases in a linear manner for 0.5N and 1N injuries. The level of corneal opacity peaks by day 3, and continues to remain high without improvement through day 21. These results demonstrate that early treatment is indicated with TSG-6, and that the efficacy of TSG-6 will be readily measureable by comparing outcomes with the untreated controls. These results are summarized in **Appendix 1, p12-14**.

3) Specific Aim 1 Subtask 3: Determine the timing and patterns of cellular changes in response to the severity of alkali injury, completed by 4/1/2015. The time course of injury data for the 0.5N and 1N injury levels were extended to day 21 for histological analysis, and for further delineation of the time course of inflammation using thrombomodulin as a marker for neovascularization, and collagen I alpha 1 as a marker for fibrosis. These markers indicate that corneal neovascularization and fibrosis increase through day 21 post injury (**Appendix 1, p15**), and is corroborated on histological examination (**Appendix 2, p17**) which demonstrates endothelial lined vascular channels in the corneal stroma with dense corneal stromal fibrosis by day 21.

The combined results of Specific Aim 1 established the proper testing conditions required to test the efficacy of TSG-6 for subsequent treatment studies. These results have been accepted as a paper presentation at the World Ophthalmology Congress, and are in preparation as a manuscript to be submitted for publication, as discussed in Accomplishments below.

4) Specific Aim 2 Subtask 1: Determine the efficacy for topical and intraocular TSG-6 after exposure to 0.5N and 1N alkali injuries, completed by end September 2015. These experiments are completed, and do not demonstrate any statistically significant benefit after 0.5N or 1N injuries for TSG-6 with regard to inflammatory cytokine message or protein, or with regard to corneal opacity for either topical application, or anterior chamber injection into the eye. These results are summarized in **Appendix 3, p19-32**. The first experiment using IV TSG-6 for treatment demonstrates a trend towards more corneal clarity at days 4 and 5, but is not statistically significant as shown in **Appendix 3, p33-38**. We believe that TSG-6 given in the intravascular space will reach viable cells better than can be achieved with topical or anterior chamber application after alkali injury and could have therapeutic benefit.

#### **c. Training Opportunities and Professional Development**

Nothing to report

#### **d. Dissemination of Findings and Results**

1) Poster presentation of time course of injury results, Annual Research Day, Central Texas Veterans Health Care System May 2015

2) Paper accepted for presentation in February 2016 at the World Ophthalmology Congress in Guadalajara Mexico titled: **Comprehensive Profiling of Alkali Injuries to the Cornea. (Appendix 4, p44.)**

3) Manuscript in preparation for publication, submission planned before the end of the calendar year.

#### **e. Plans for Next Reporting Period**

1) We plan begin the testing of the efficacy of intravascular TSG-6 in the treatment of corneal alkali injuries. We will amend the SOW to eliminate the therapeutic window of topically applied TSG-6 because we have not been able to demonstrate efficacy of topical TSG-6, and efforts will henceforth focus on intravascular application with increased dosing.

2) The quantity of TSG-6 that is required for IV treatment is higher than that for topical or anterior chamber application, and we are producing and purifying TSG-6 in the large quantities required to accomplish the treatment in an efficient and cost effective manner.

3) An amendment to the protocol will be submitted this quarter for approval by all agencies to include the IACUC, ACURO, and Central Texas Veterans Health Care System research committees. The amendment will incorporate proposed changes to the SOW once approved by CDMRP, and will reflect an expansion of intravascular testing and increased dosing of TSG-6, while eliminating the determination of the therapeutic window for topical application of TSG-6.

#### **4. IMPACT**

##### **a. Principal Discipline**

The results of this work for this report are the most encompassing and complete of any other reported study that we are aware of for chemical alkali injuries to the cornea, and will serve the ophthalmic community in the future as a benchmark model. The complete description of time course of injury with inflammatory cytokine and protein expression, clinical evaluation, and histopathology correlates to 21 days post injury is not duplicated in the literature, and is why we believe our abstract was accepted as a paper presentation at the prestigious World Ophthalmology Congress.

##### **b. Other Disciplines**

The results of this work, albeit with lack of demonstrated efficacy of TSG-6 to date with topical or anterior chamber application, will help investigators in the field of Regenerative Medicine focus on alternate methods of delivery, and show that while TSG-6 has been shown to be effective in a model of mild chemical corneal injury induced by ethanol, it is insufficient to benefit more severe ocular alkali chemical injuries when applied topically or in the anterior chamber.

##### **c. Technology Transfer**

Nothing to report

##### **d. Impact on Society**

Nothing to report

#### **5. CHANGES/PROBLEMS**

##### **a. Changes**

1) During the reporting period, we eliminated the 0.1N injury level due to the minimal amount of injury observed in early experiments which would not allow potential treatment benefit to be observed. We also expanded the time course endpoints for the 0.5N and 1N injury levels to conclusively determine whether or not TSG-6 application would be beneficial in the early and late stages of the inflammatory response, and expanded the anterior chamber treatment to include the moderate and severe injury levels. The amended protocol was approved IACUC on 4/17/2015, and subsequently by ACURO on 4/27/2015. The revised SOW to reflect these changes was approved by CDMRP in April 2015.

**b. Actual/Anticipated Problems**

1) There were no actual problems that resulted in delays for this reporting period

2) We anticipate potential logistical issues in the coming quarter that relate to the move that is required of Dr. Prockop and the Institute of Regenerative Medicine to College Station from Temple, Texas by Texas A&M. Inasmuch as Dr. Prockop and his team are consultants on our project, and we have been sharing his laboratory space and equipment, as well as the TSG-6 that his team produces and purifies, his move has potential to impact our study. Fortunately, we have been given ample laboratory space in the Research facility at Central Texas Veterans Health Care System (CTVHCS) in Building 205 Room 3R34, and CTVHCS is actively procuring all the equipment that we will need to continue our project. We have transferred protein production and purification to our team, and included in **Appendix 3, p39-42** are the initial yields of TSG-6 produced from CHO cell lines, and which will result in over 100 mg of purified protein TSG-6 at a very low cost. Dr. Prockop and his team will continue to collaborate as consultants even with their move to College Station, and we will continue our weekly meetings with Skype or conference calls.

**c. Changes in Expenditures**

Nothing to Report

**d. Changes in Animal Use**

No changes to report other than that reported under **a.** above

**6. PRODUCTS**

**a. Publications/papers/presentations**

1) Poster presentation **Comprehensive Profiling of Alkali Injuries to the Cornea**, presented at the Annual Research Symposium at the Central Texas Veterans Health Care System, spring 2015.

2) Paper presentation accepted, to be presented February 2016, **Comprehensive Profiling of Alkali Injuries to the Cornea**, at the World Ophthalmology Congress, Guadalajara Mexico

3) Manuscript in preparation for submission by end of calendar year, to be completed after analysis of histochemical slides, **Comprehensive Profiling of Alkali Injuries to the Cornea**

**b. Websites**

1) Central Texas Veterans Research Foundation <http://www.ctvrf.org/research-programs>

**c. Technologies/Techniques**

Nothing to report

**d. Inventions/Patents/Licenses**

Nothing to report

**7. PARTICIPANTS/COLLABORATING ORGANIZATIONS**

**a. Individuals**

1) Name: Hosoon Choi, PhD  
Project Role: Research Scientist  
Researcher Identifier (e.g. ORCID ID): NA  
Nearest person month worked: 11  
Contribution to Project: Dr. Choi has performed rat surgery to establish injury levels, performed chemokine and cytokine assays, and protein production and purification

2) Name: Casie Phillips

Project Role: Animal Technician, research assistant  
Researcher Identifier (e.g. ORCID ID): NA  
Nearest person month worked: 10

Contribution to Project: Casie has assisted Dr. Choi in animal surgery, performs postoperative care, assists with laboratory techniques, prepares and stains histology specimens

3) Name: Samuel Fulcher, MD  
Project Role: PI  
Researcher Identifier (e.g. ORCID ID): NA  
Nearest person month worked: 12  
Contribution to Project: Dr. Fulcher performs the duties of the PI, and scores the clinical injuries by grading photographs of the corneal opacity

#### **b. Collaborating Institutions**

1) Texas A&M Institute for Regenerative Medicine, Temple, Texas. Dr. Darwin Prockop and his team have served as unpaid consultants, and shared bench laboratory space with our team.

2) Central Texas Veterans Research Foundation, Temple, Texas. CTVRF is the sponsoring agency

3) Central Texas Veterans Health Care System, Temple, Texas. CTVHCS is the employer of Dr. Fulcher, and has allotted time for research activities, and is supporting the project with new laboratory space and equipment

4) Baylor Scott and White Health Care, Temple, Texas. BSW is the sponsor of the IACUC, and operates and maintains the vivarium where experiments are performed, and employs the supervising veterinarian who oversees all animal research projects.

#### **8. SPECIAL REPORTING REQUIREMENTS**

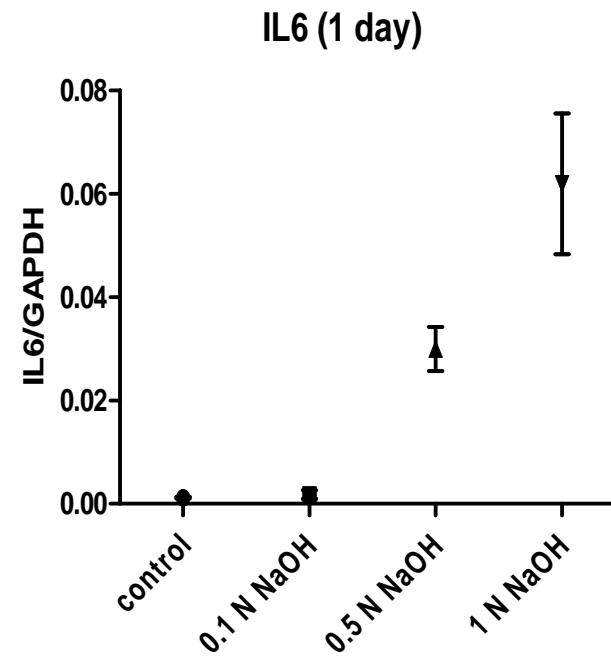
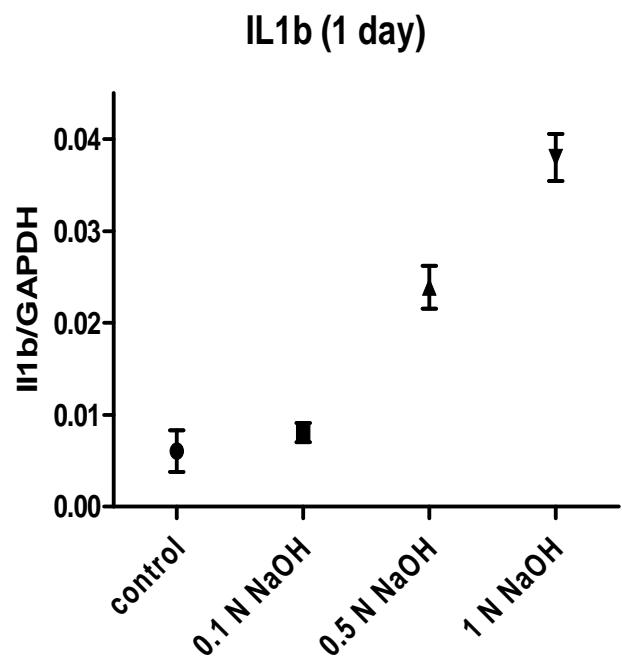
**a. No Collaborative Awards to report**

**b. Quad Chart, (Appendix 5, p46.)**

#### **9. APPENDICES, SEE APPENDICES A1-A5**

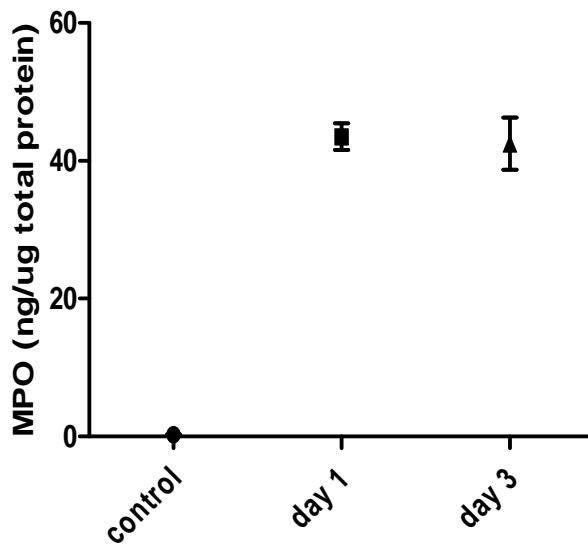
# Appendix 1

## NaOH concentration dependent increase of cytokine expression

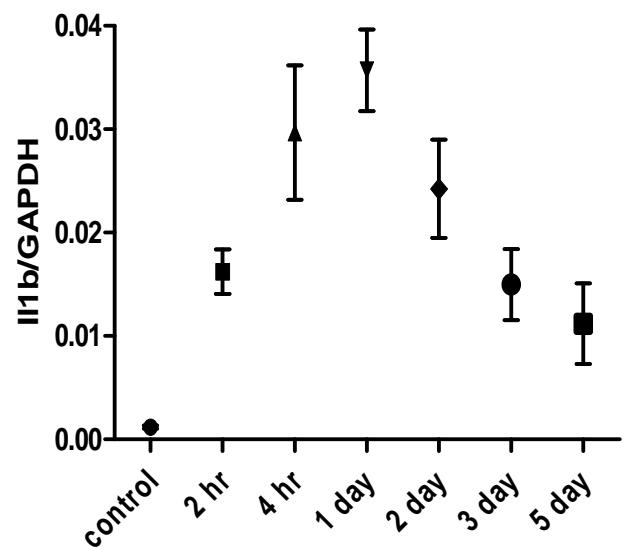


# 1 N NaOH time course

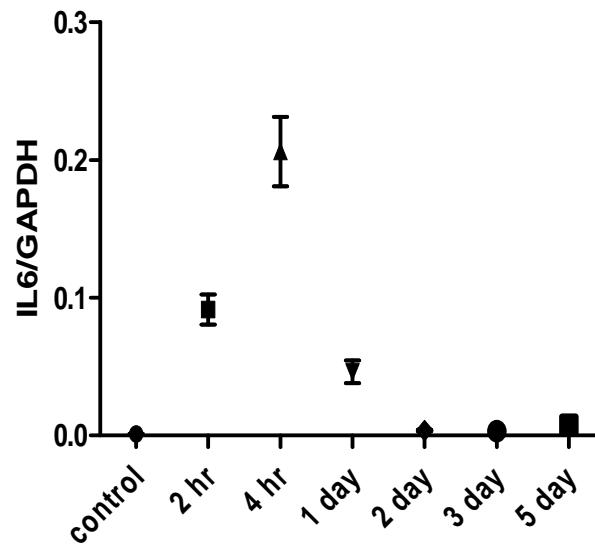
MPO (1 N NaOH)

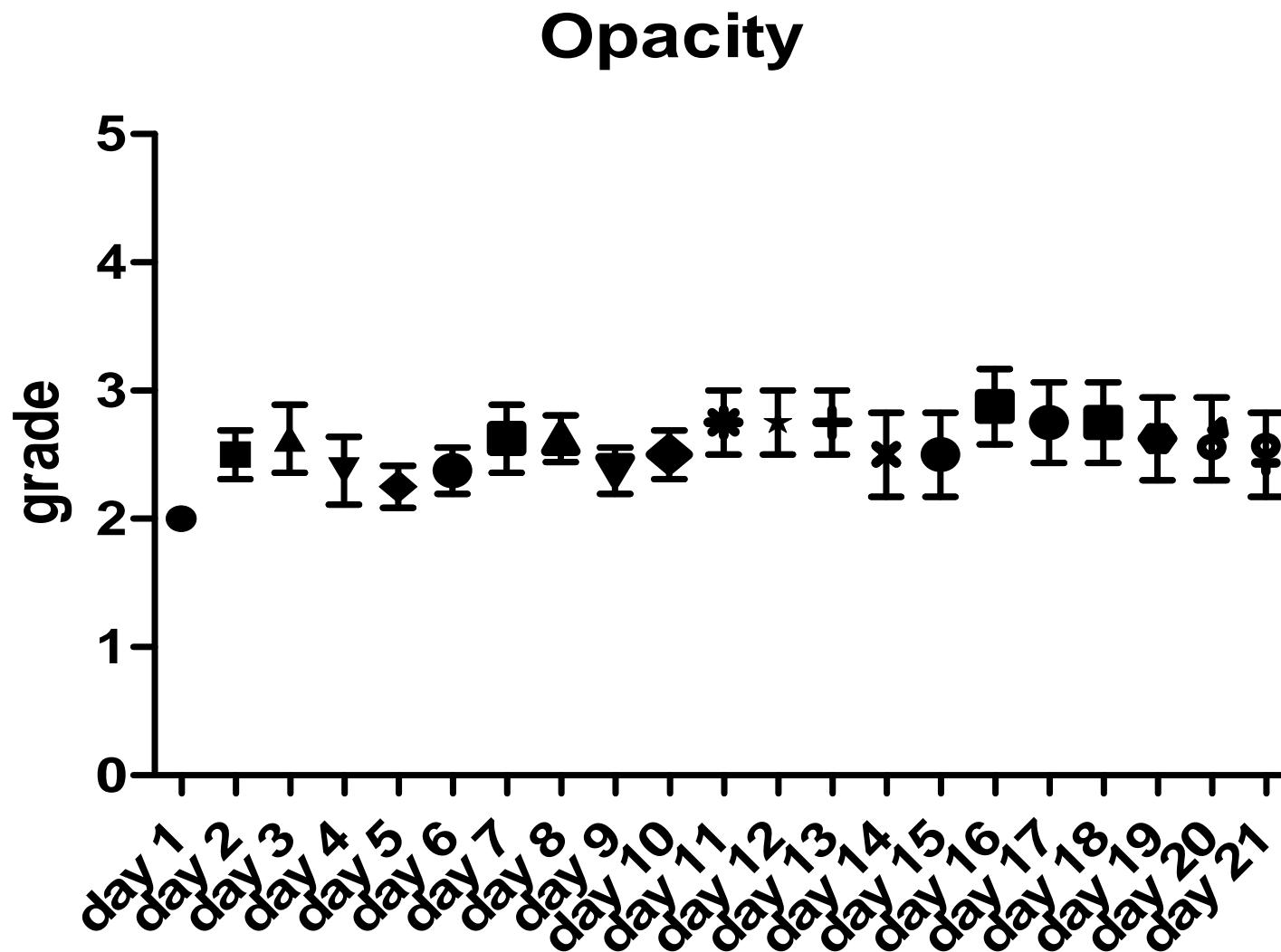


IL1b (1N NaOH)

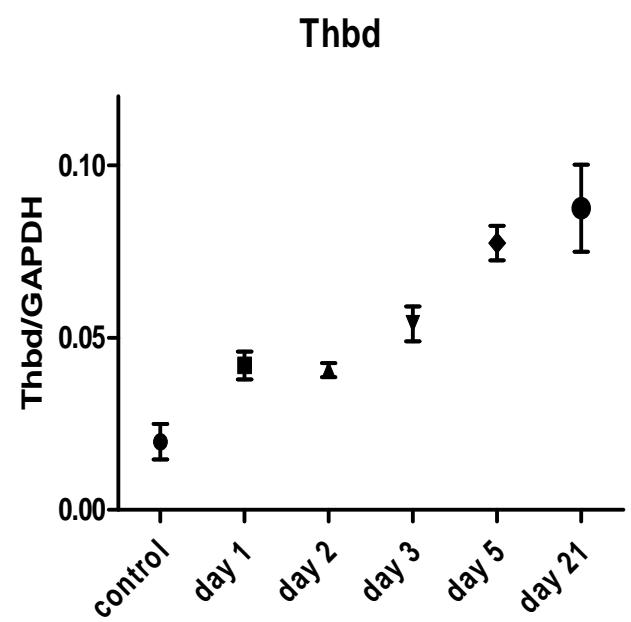


IL6 (1N NaOH)

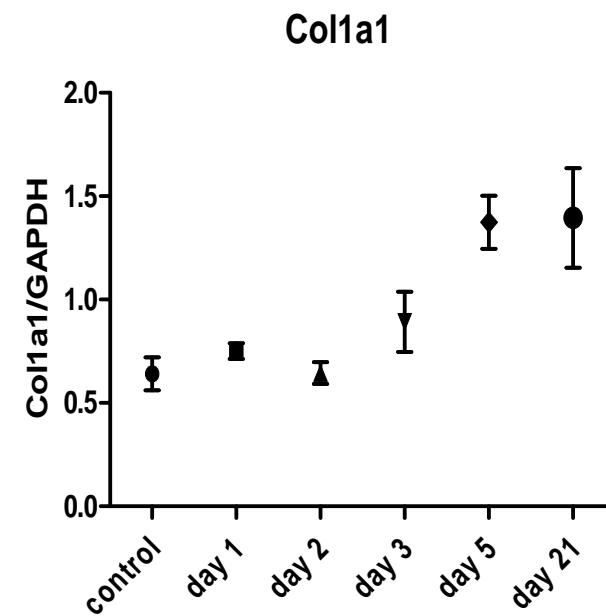




## Neovascularization and fibrosis by 1 N NaOH injury

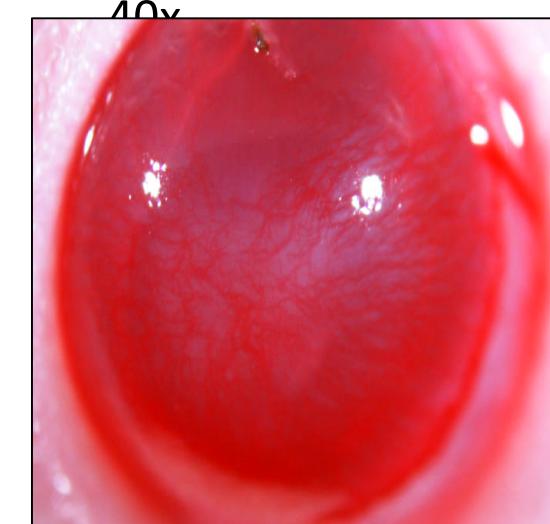
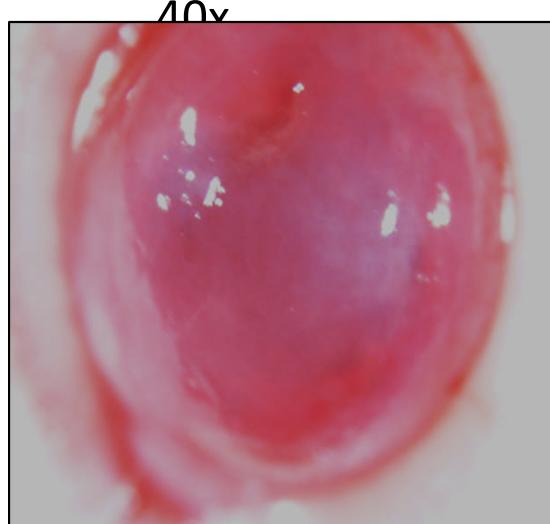
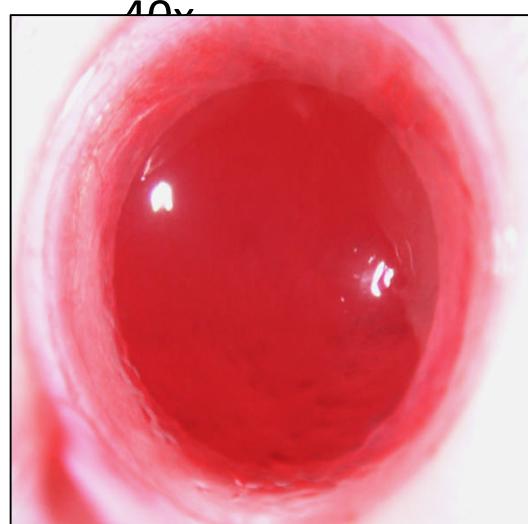
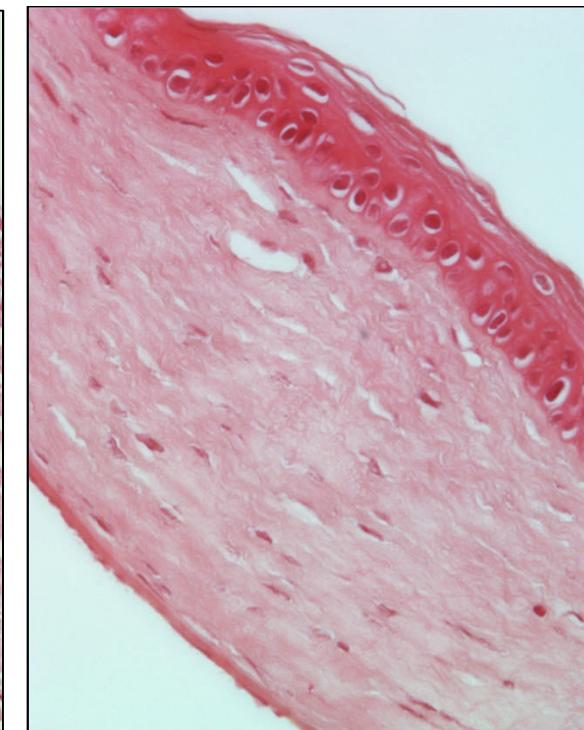
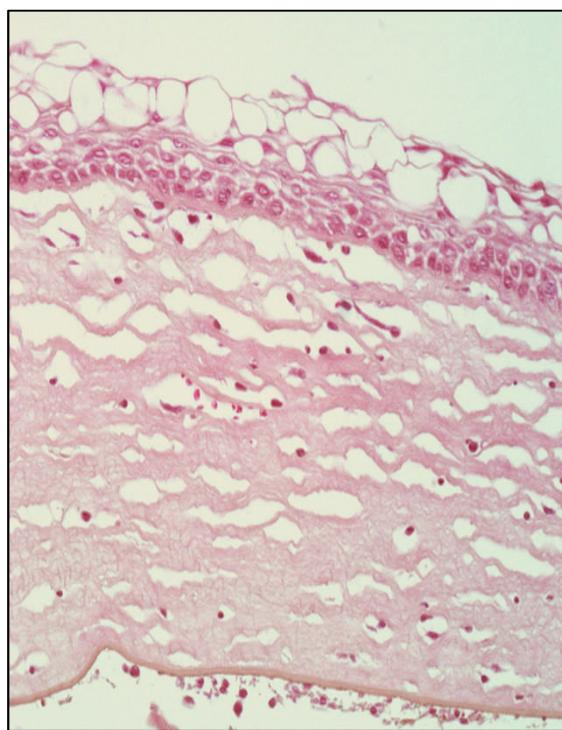
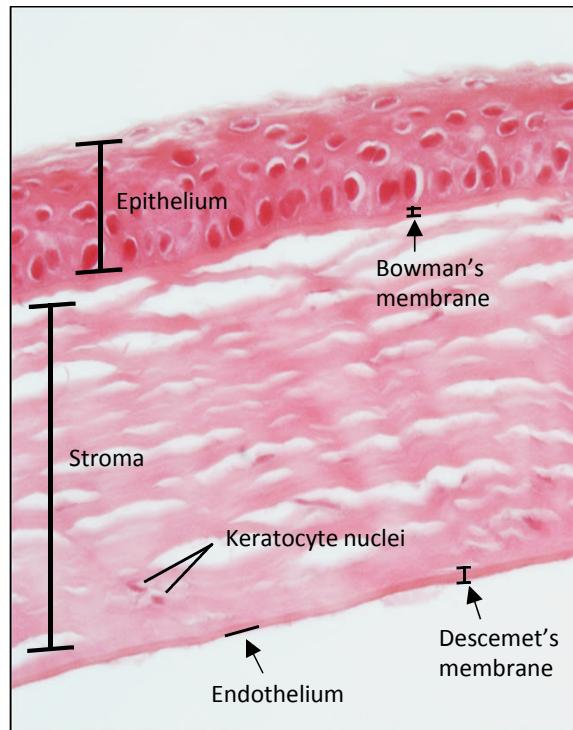


Thbd (thrombomodulin)  
alpha 1



Col1a1 (collagen, type I,

# Appendix 2



# Appendix 3

# Corneal Injury Model

## AC Injection: TSG-6 / PBS

0.5 N NaOH (30 sec) ; PBS Flush 50 mL

2014-013-R

- **Date of study:**

6-5-15 through 6-12-15

- **Number of animals used:**

8 rats with 0.5 N NaOH injury + TSG-6 AC injection (5 uL; 0.5 ug/uL)

8 rats with 0.5 N NaOH injury + PBS AC injection (5 uL)

= 16 Lewis rats total

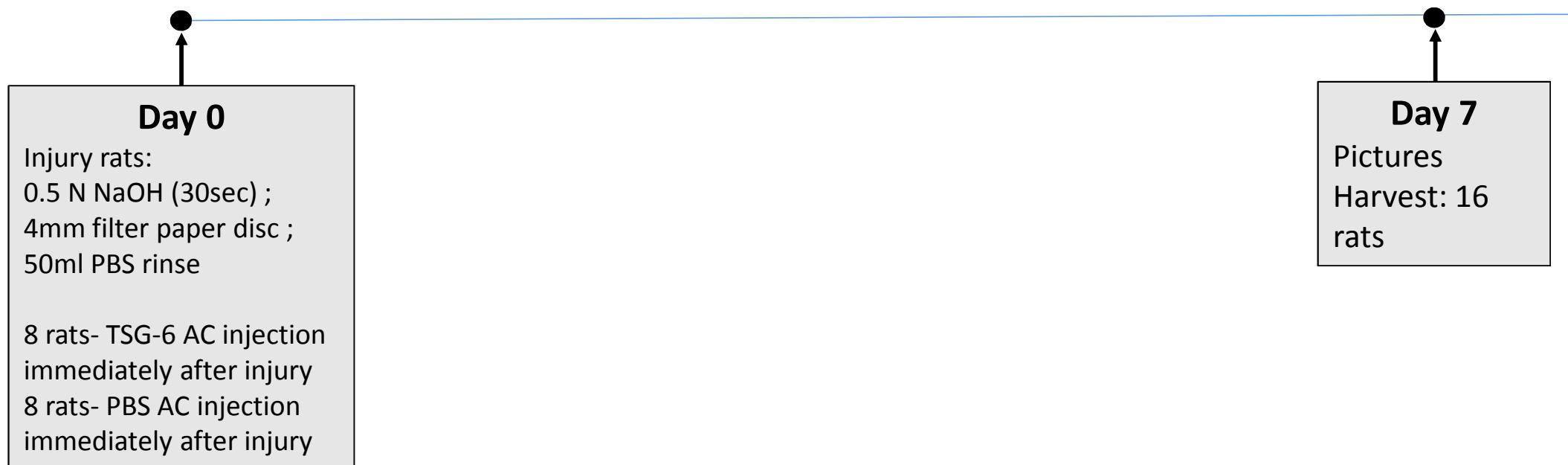
- **Harvest day / number of animals:**

Day 7 harvest / 16 rats



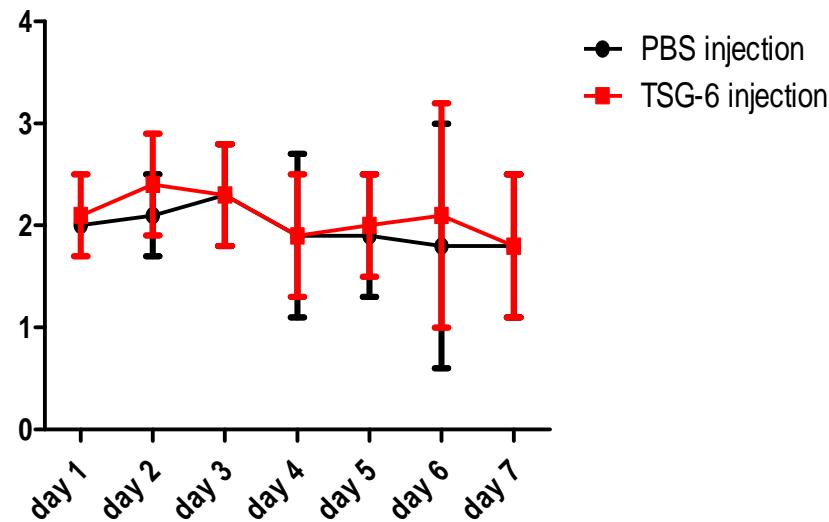
# Alkali Injury of Rat Cornea

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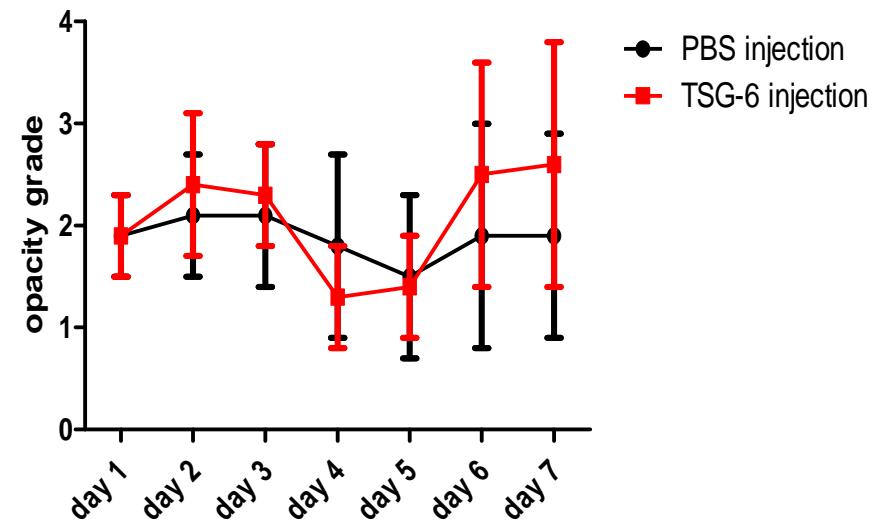


\* Photographic data was recorded daily for each rat until time of harvest

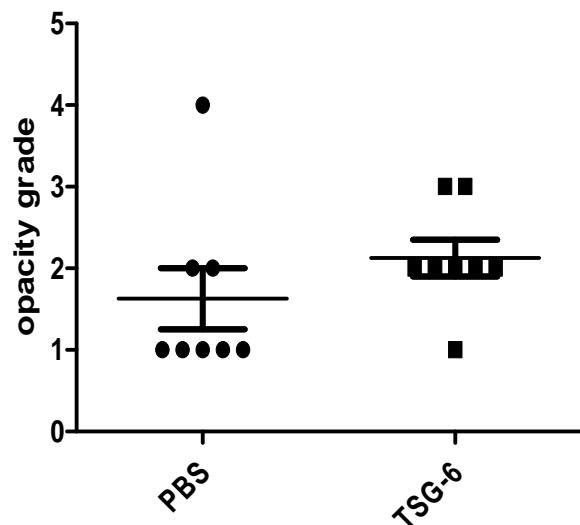
Opacity (Dr. Fulcher)

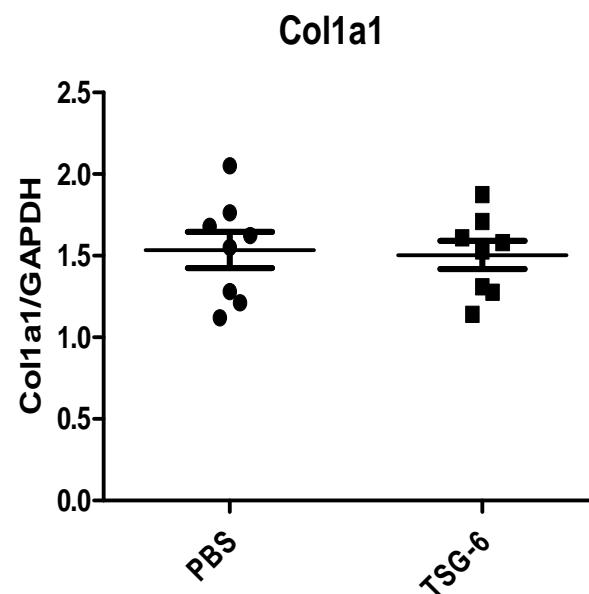
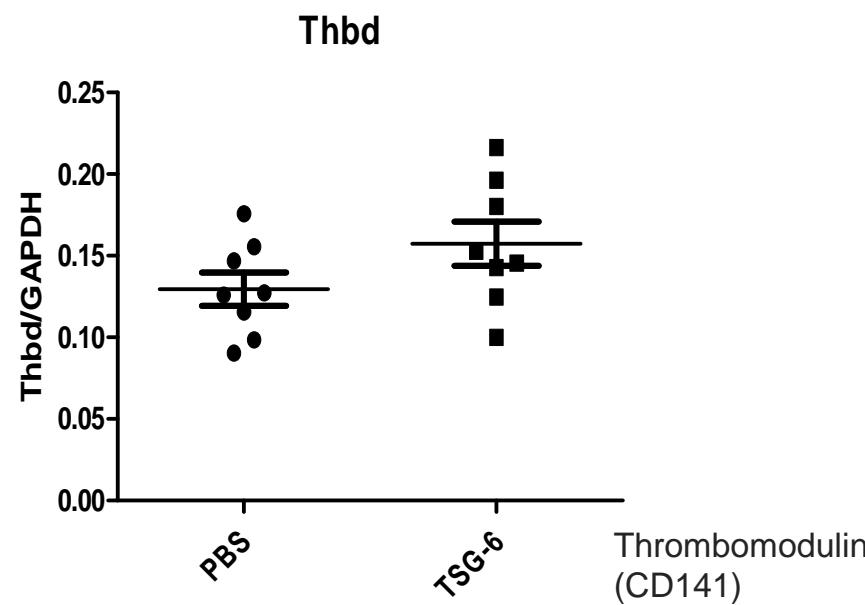
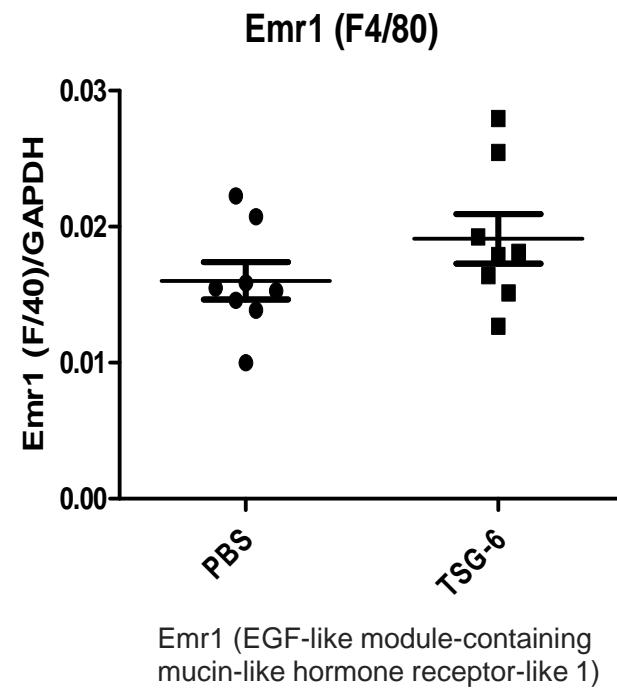
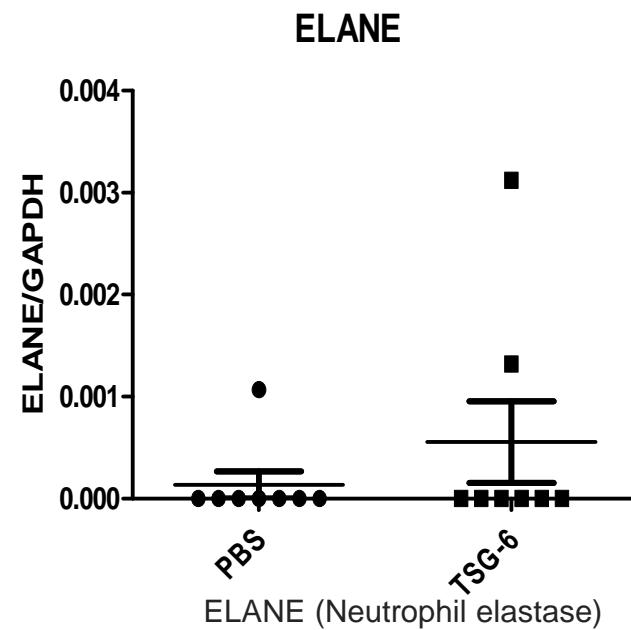
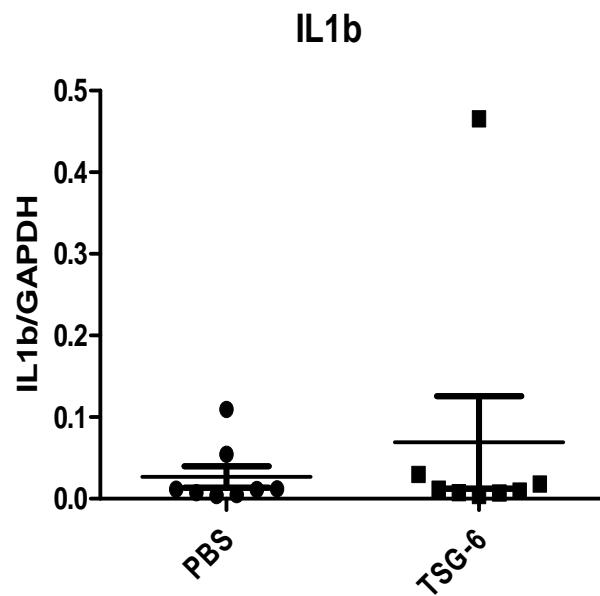


Opacity (Dr. Oh)



Opacity (microscope) day 7





Corneal Injury Model  
AC Injection: TSG-6 / PBS  
1 N NaOH (30 sec) ; PBS Flush 50 mL  
2014-013-R

- **Date of study:**

6-19-15 through 6-26-15

- **Number of animals used:**

8 rats with 1 N NaOH injury + TSG-6 AC injection (5  $\mu$ L; 0.5  $\mu$ g/ $\mu$ L)

7 rats with 1 N NaOH injury + PBS AC injection (5  $\mu$ L)

= 15 Lewis rats total

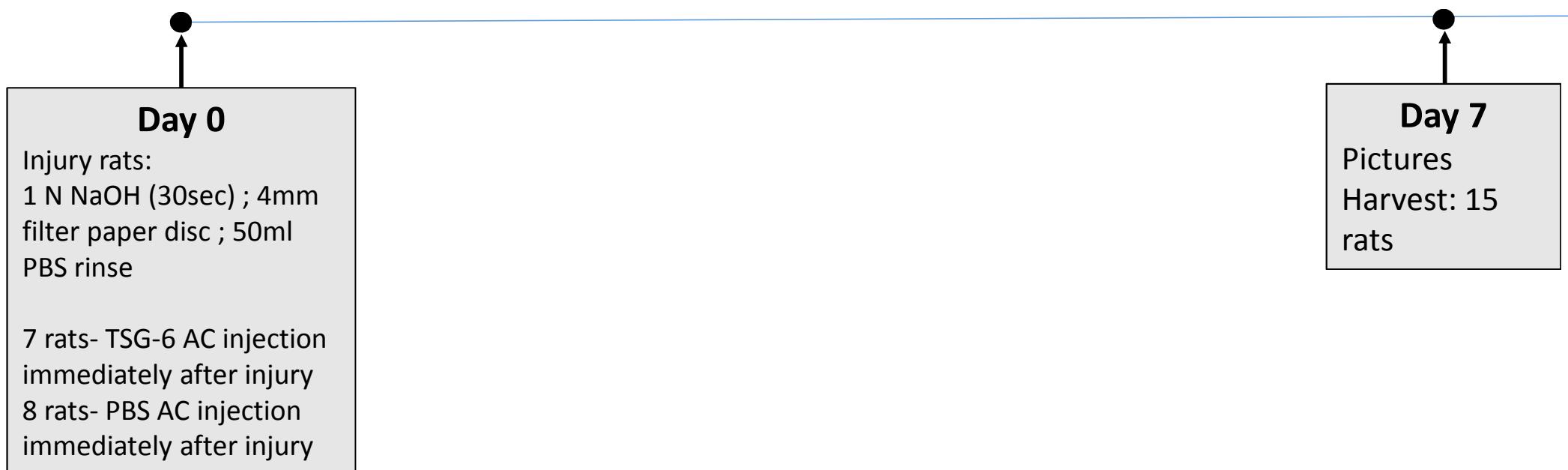
- **Harvest day / number of animals:**

Day 7 harvest / 15 rats

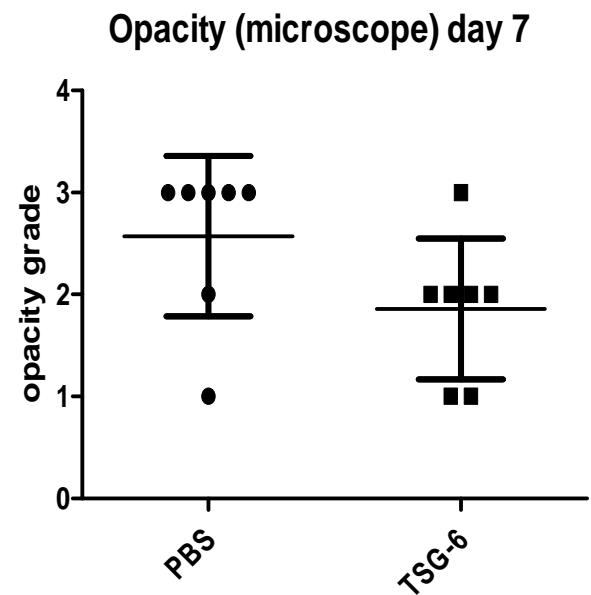
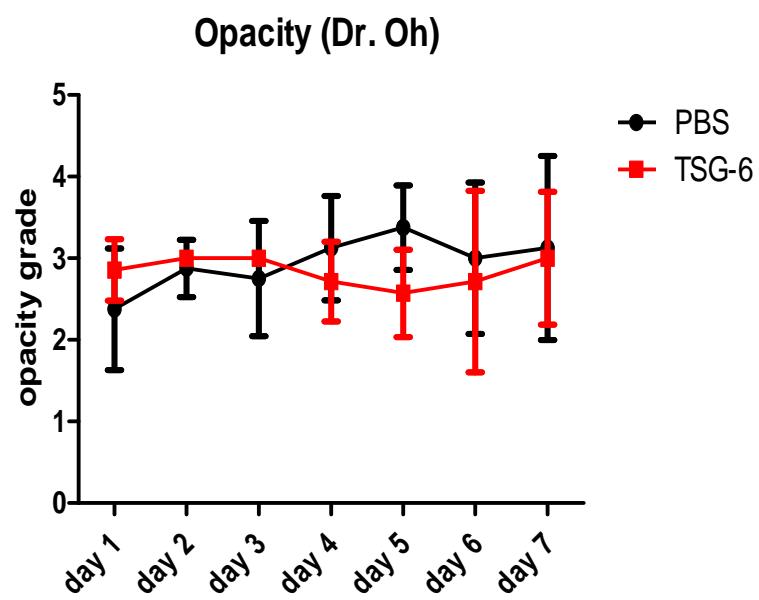
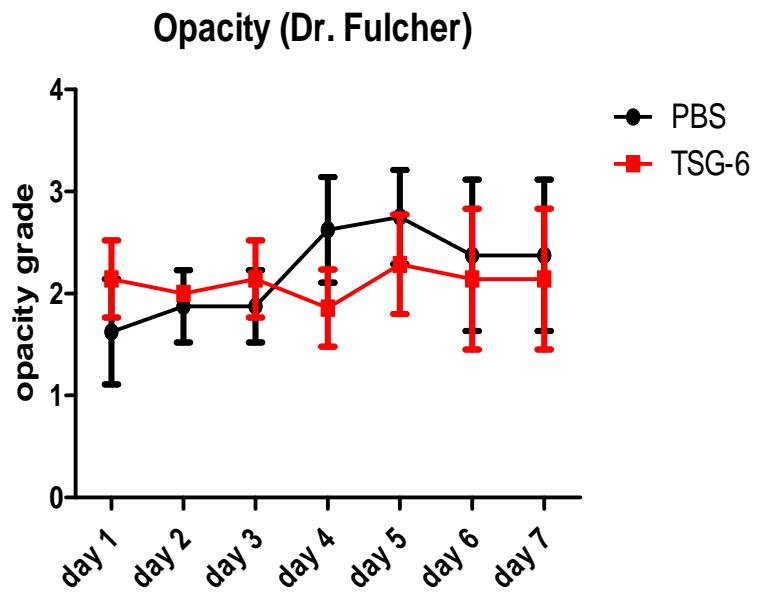


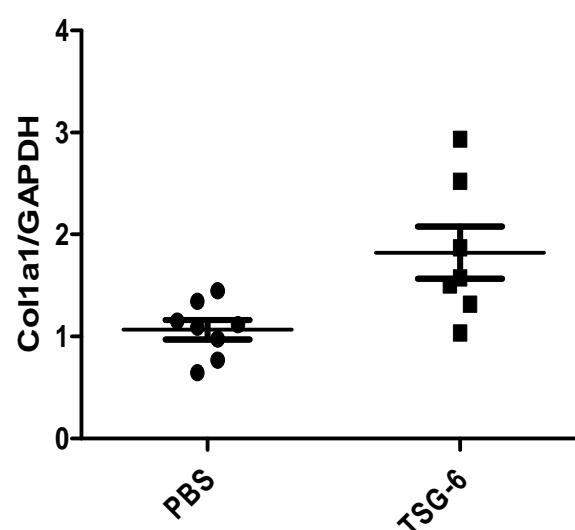
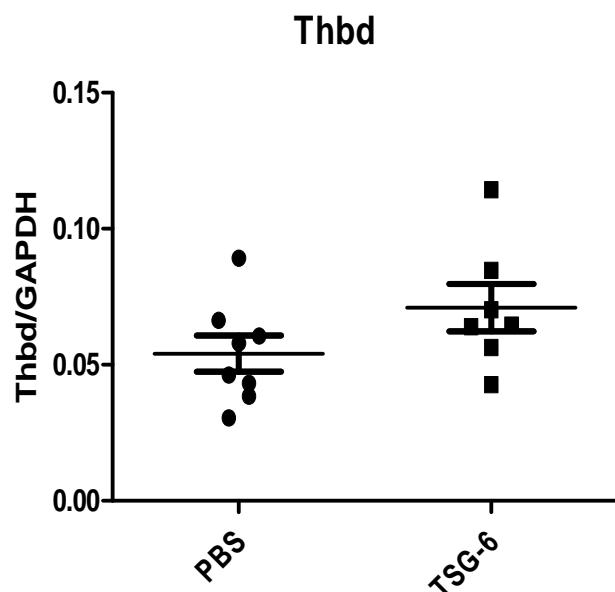
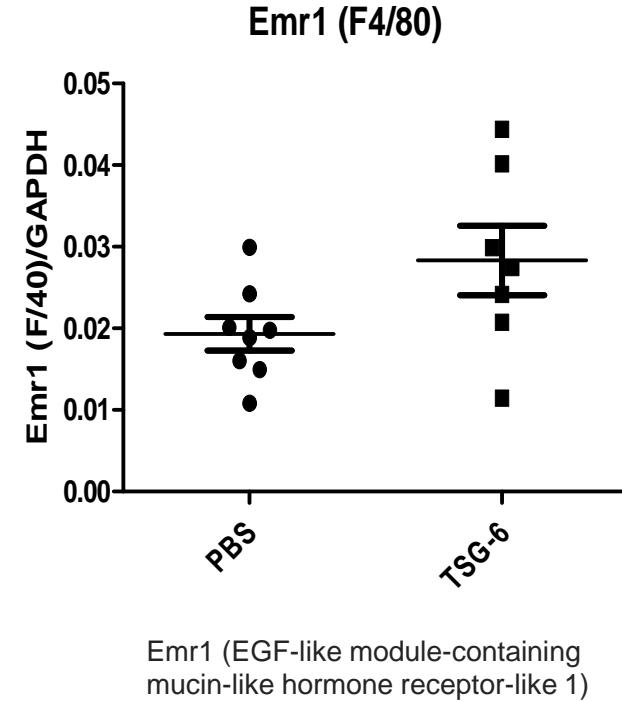
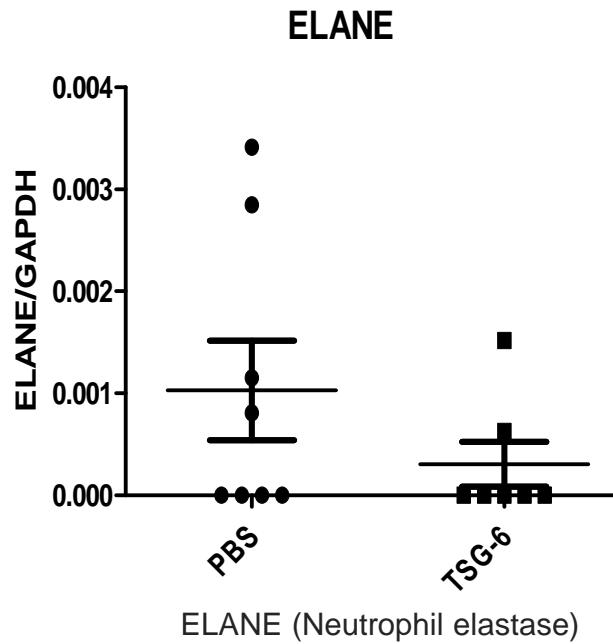
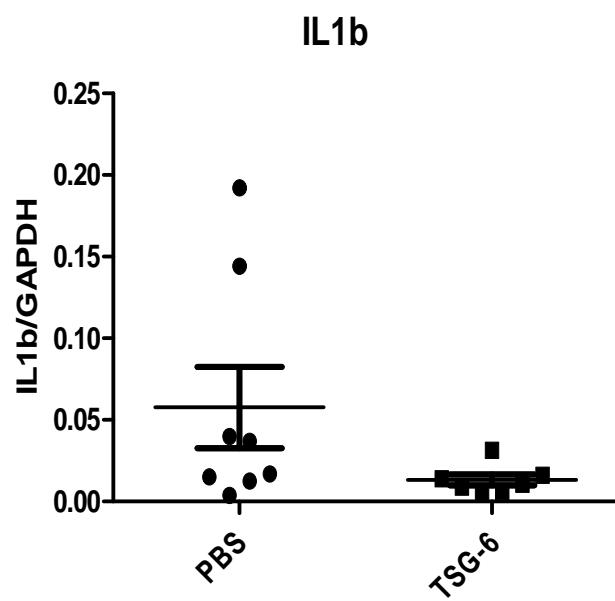
# Alkali Injury of Rat Cornea

data reflects 6/19-26



\* Photographic data was recorded daily for each rat until time of harvest





# Corneal Injury Model

Topical: TSG-6 / PBS

1.0 N NaOH (30 sec) ; PBS Flush 50 mL

2014-013-R

- **Date of study:**

8-6-15 through 8-13-15

- **Method of Treatment:**

Silicone ring (30 min) filled w/TSG-6 or PBS immediately following injury, then 5  $\mu$ l drop three times per day (TID)

- **Number of animals used:**

8 rats with 1.0 N NaOH injury + TSG-6 topical

8 rats with 1.0 N NaOH injury + PBS topical

= 16 Lewis rats total

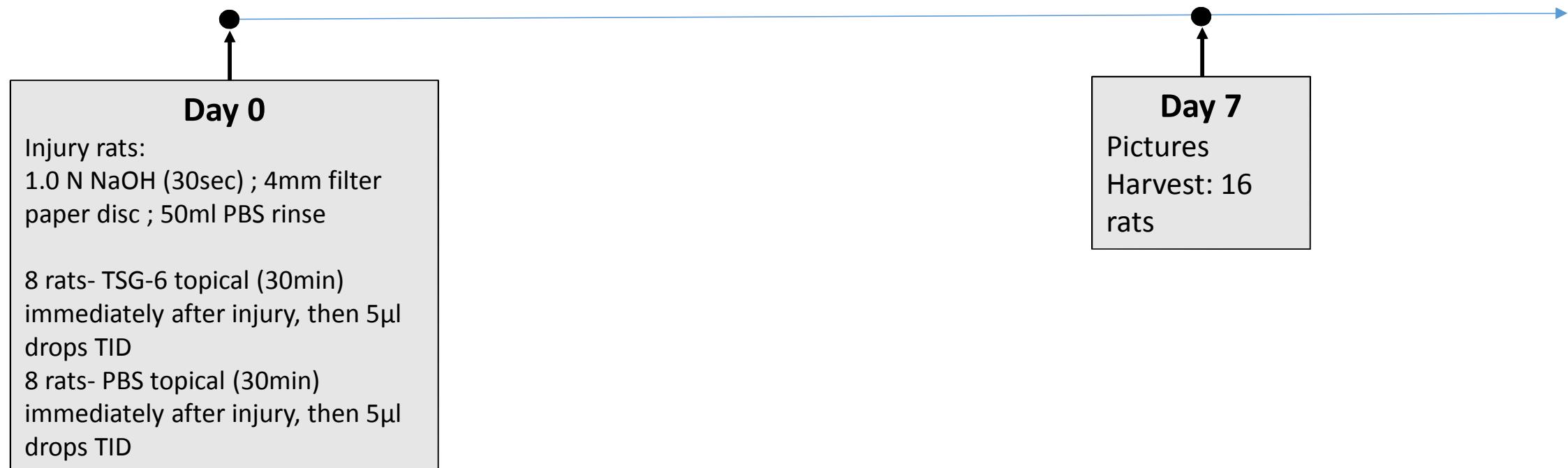
- **Harvest day / number of animals:**

Day 7 harvest / 16 rats

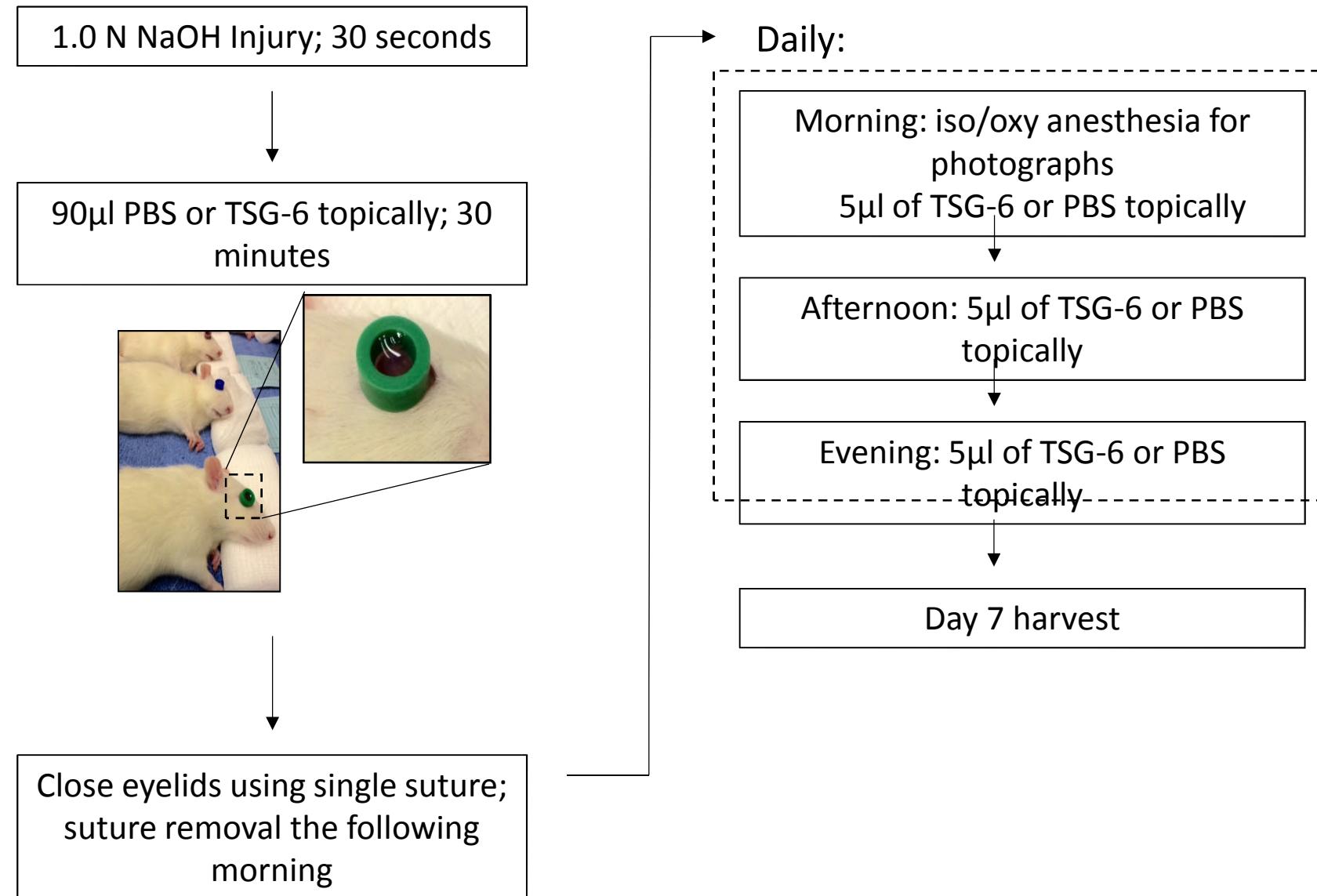


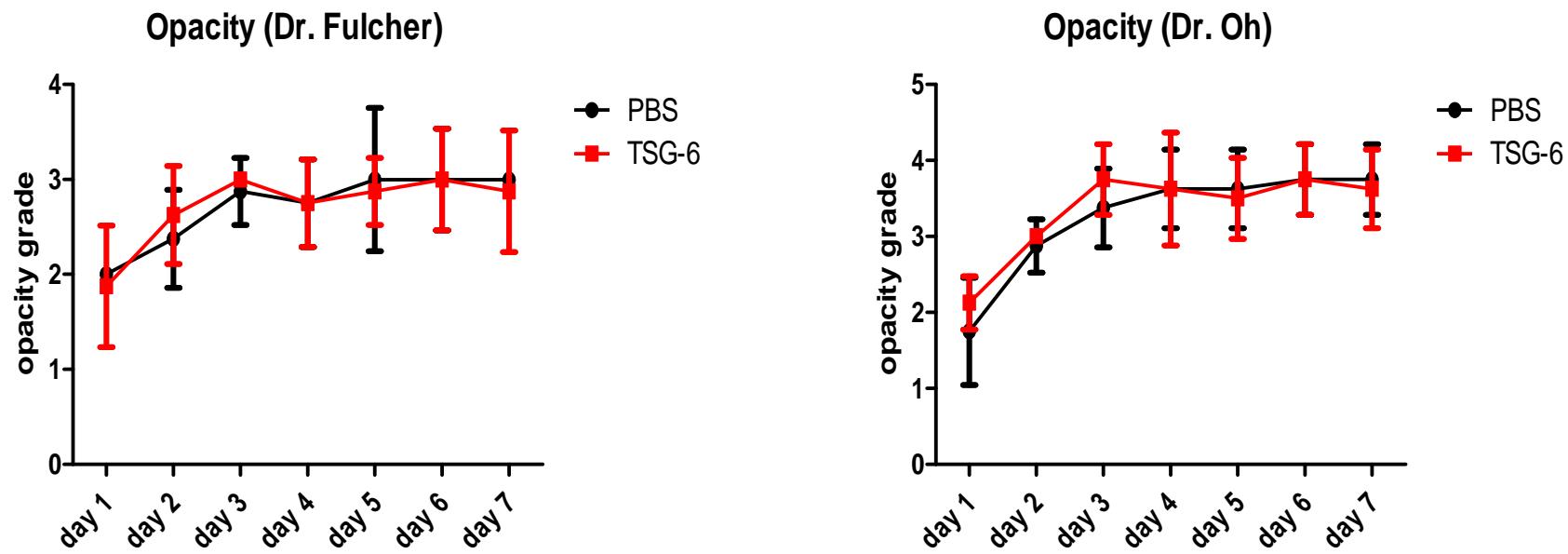
# Alkali Injury of Rat Cornea

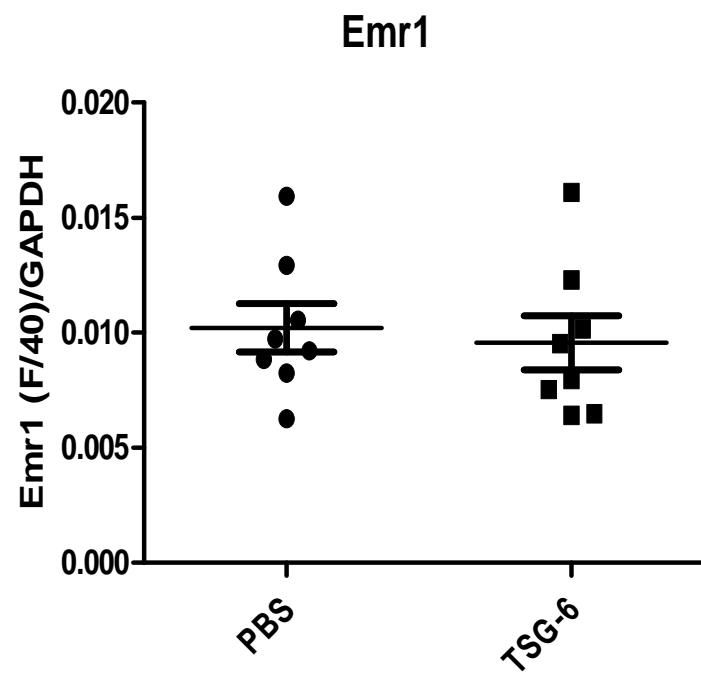
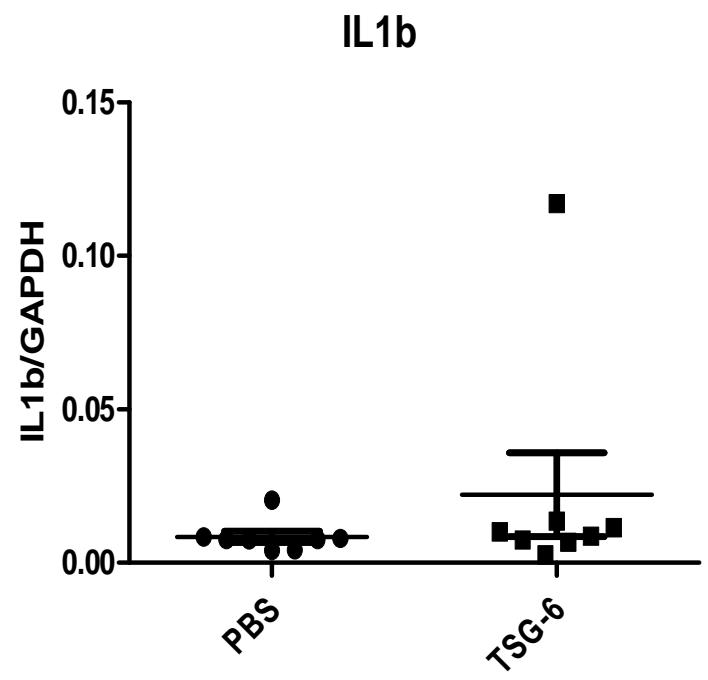
data reflects 8-6-15 injury date

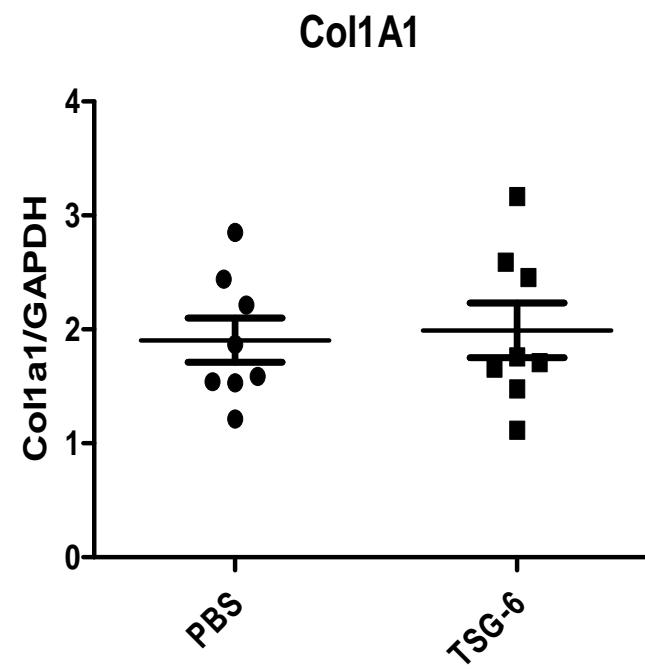
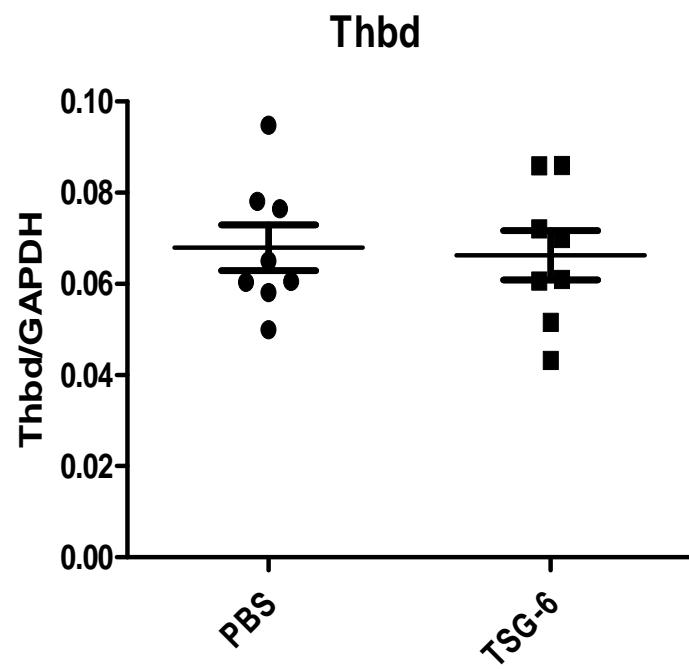


\* Photographic data was recorded daily for each rat until time of harvest









# Corneal Injury Model

## IV Injection: TSG-6 / PBS

1.0 N NaOH (30 sec) ; PBS Flush 50 mL

2014-013-R

- **Date of study:**

7-7-15 through 7-12-15

- **Number of animals used:**

7 rats with 1.0 N NaOH injury + TSG-6 IV injection (200 ug/rat)

7 rats with 1.0 N NaOH injury + PBS IV injection (264 uL)

= 14 Lewis rats total

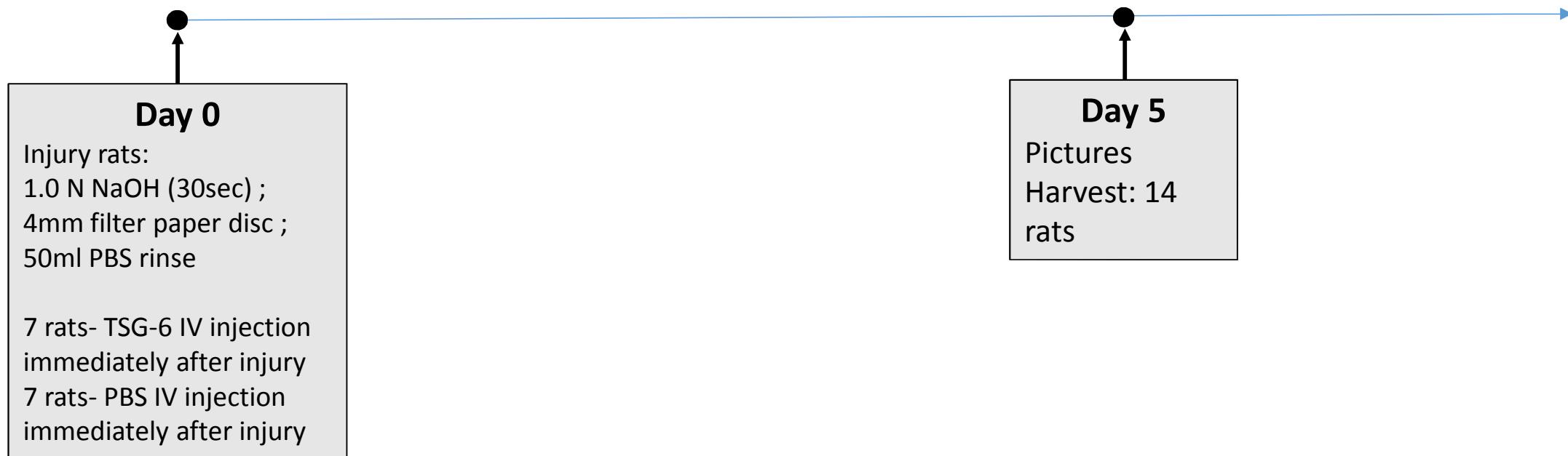
- **Harvest day / number of animals:**

Day 5 harvest / 14 rats

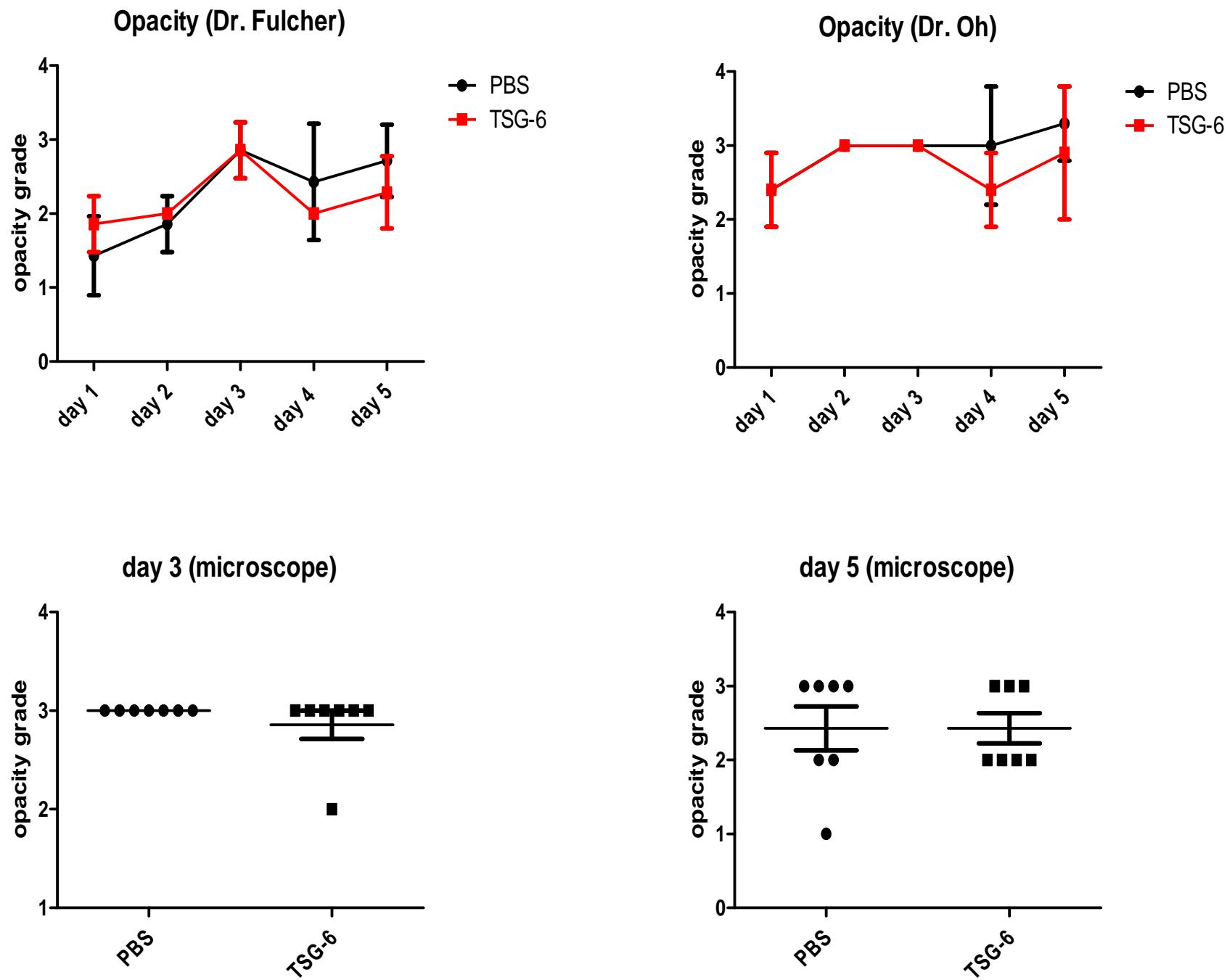


# Alkali Injury of Rat Cornea

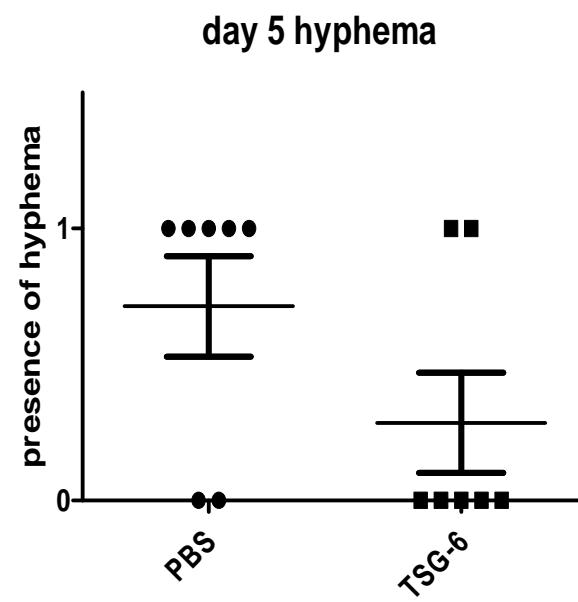
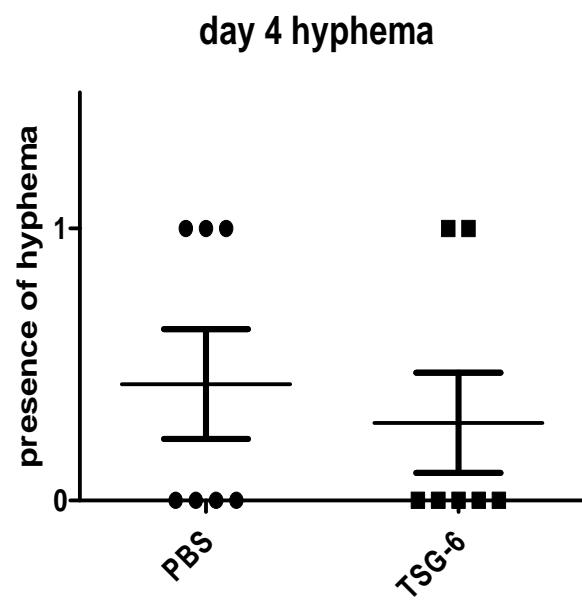
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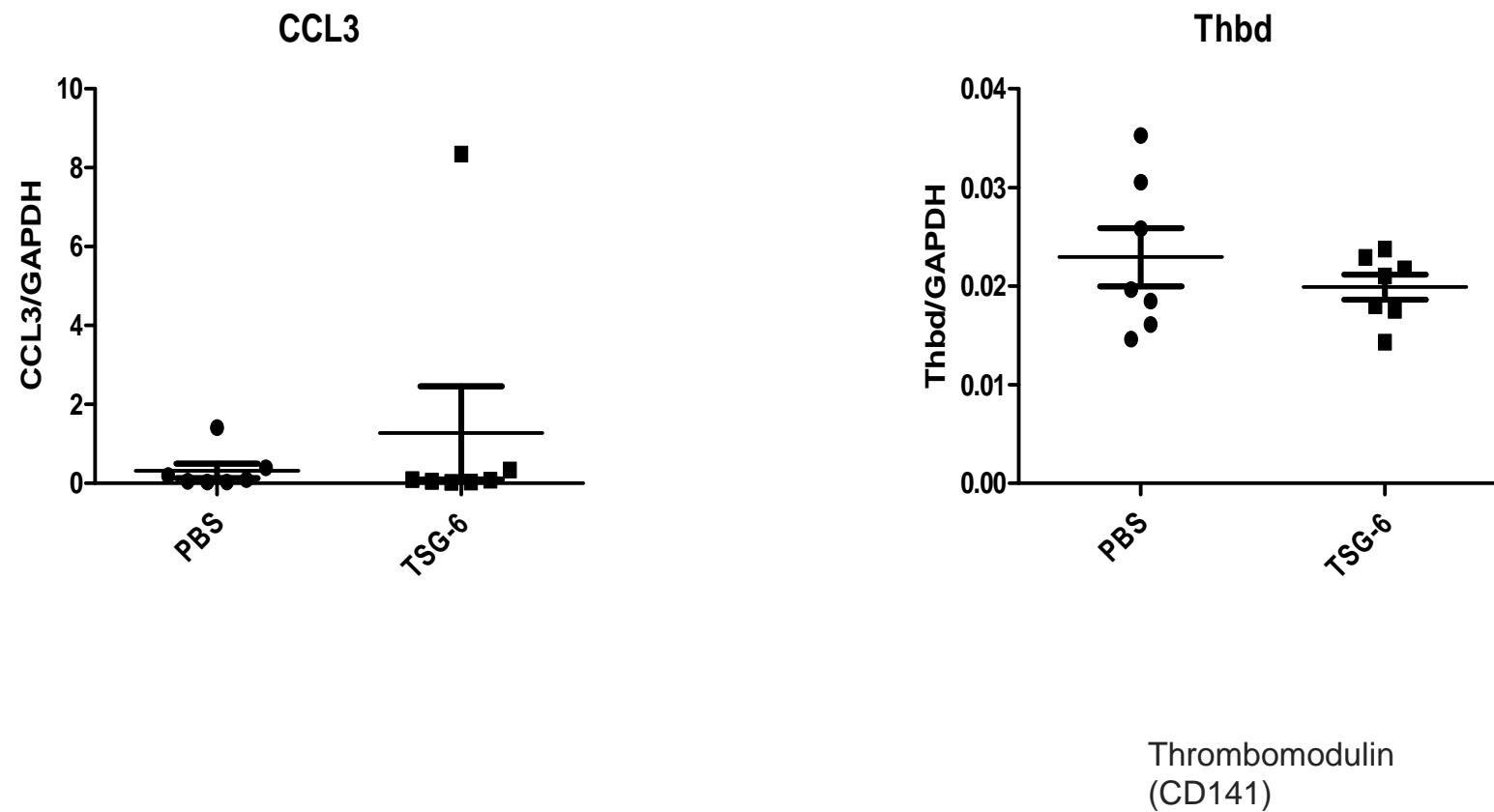


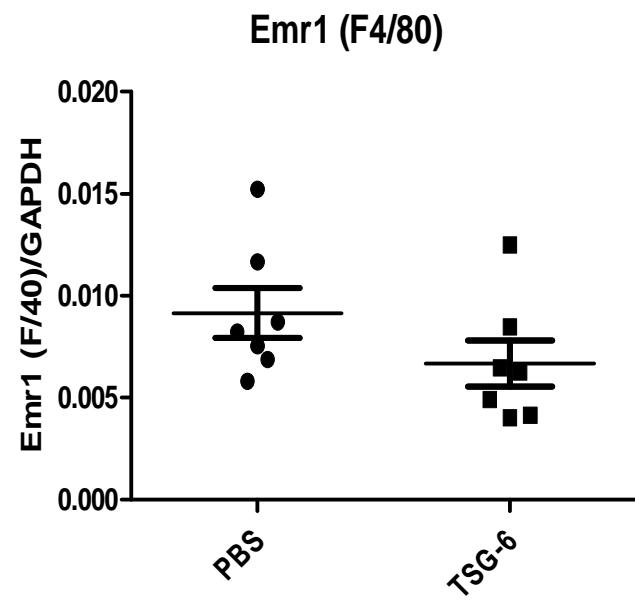
\* Photographic data was recorded daily for each rat until time of harvest



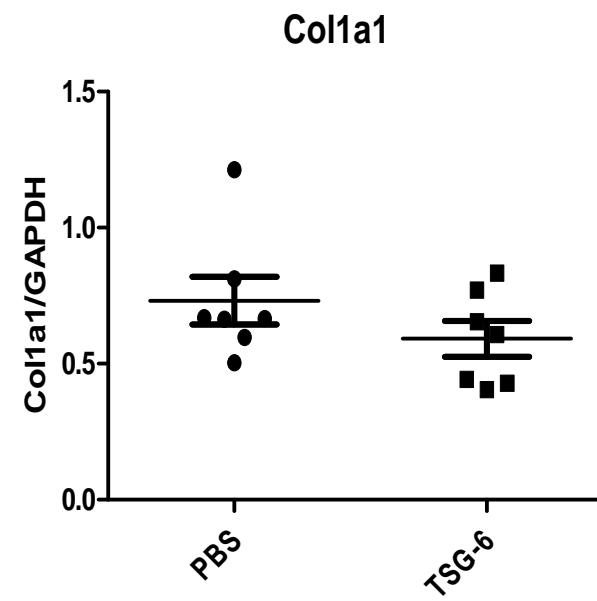
1N NaOH, IV, day 5

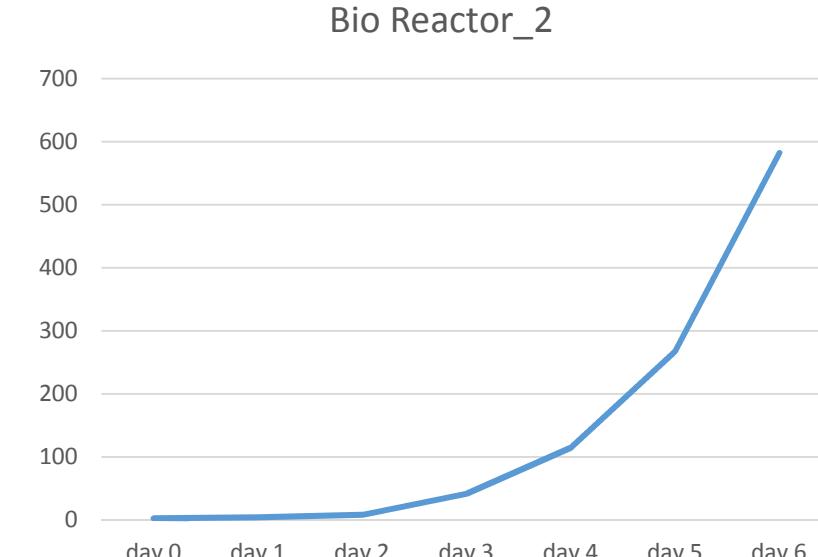
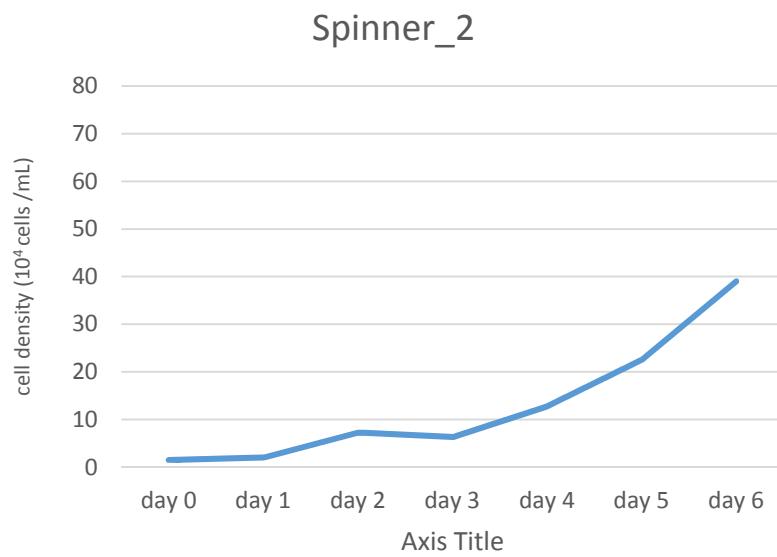
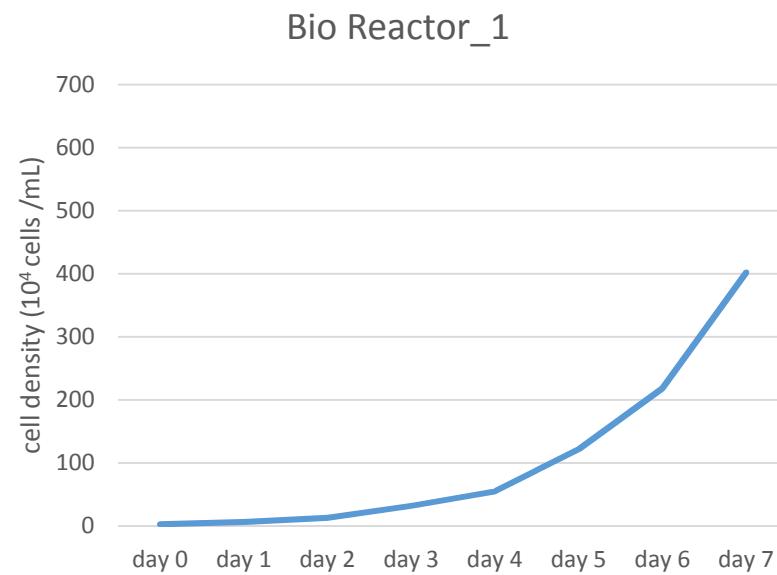
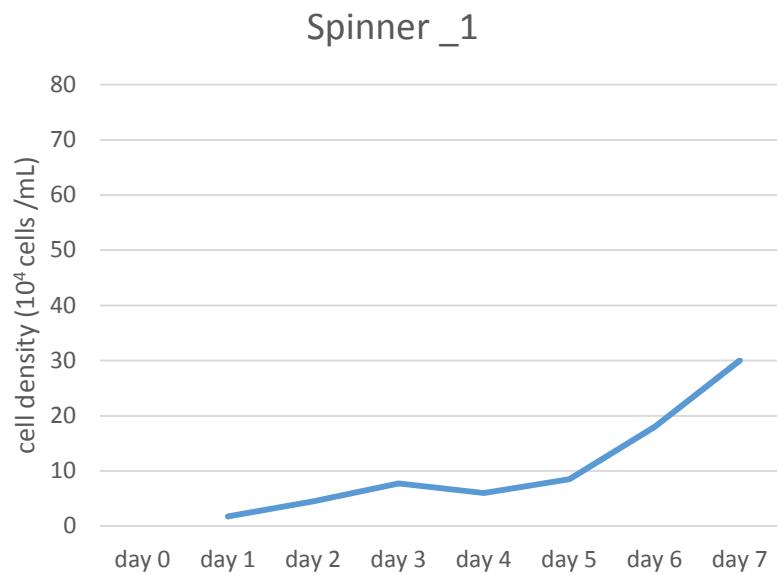




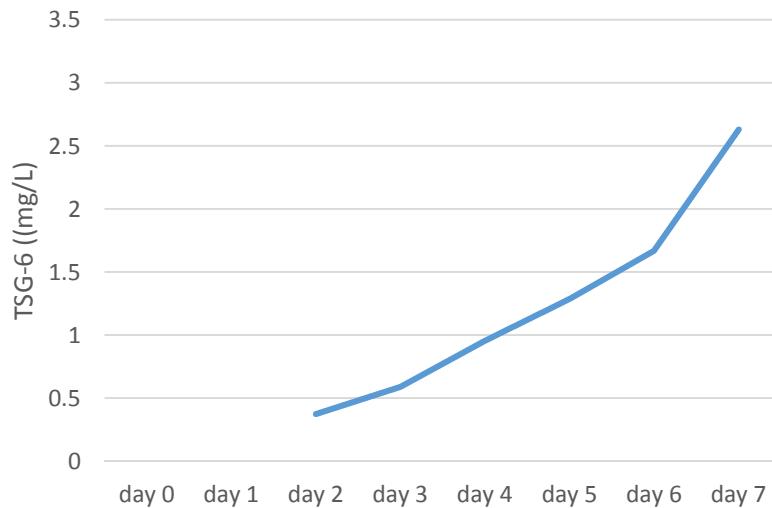


Emr1 (EGF-like module-containing  
mucin-like hormone receptor-like 1)

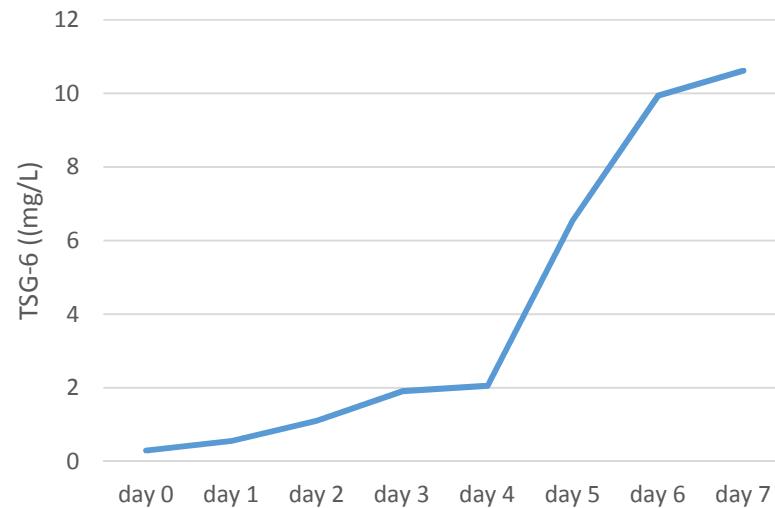




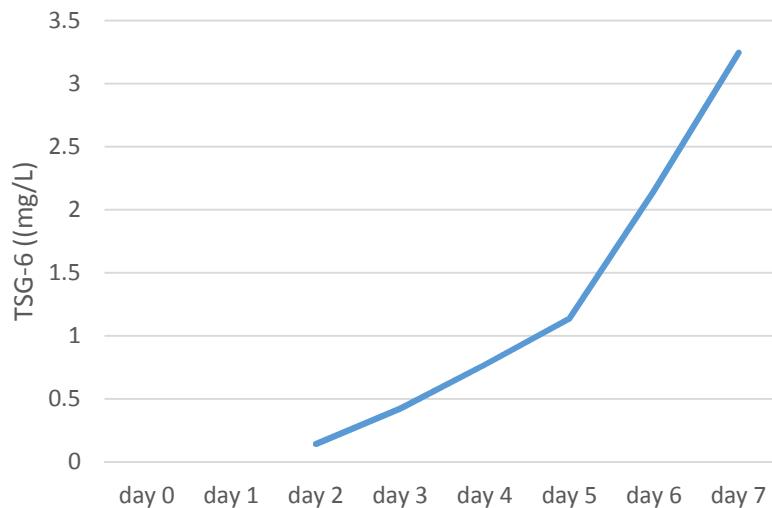
TSG-6 (Spinner\_1)



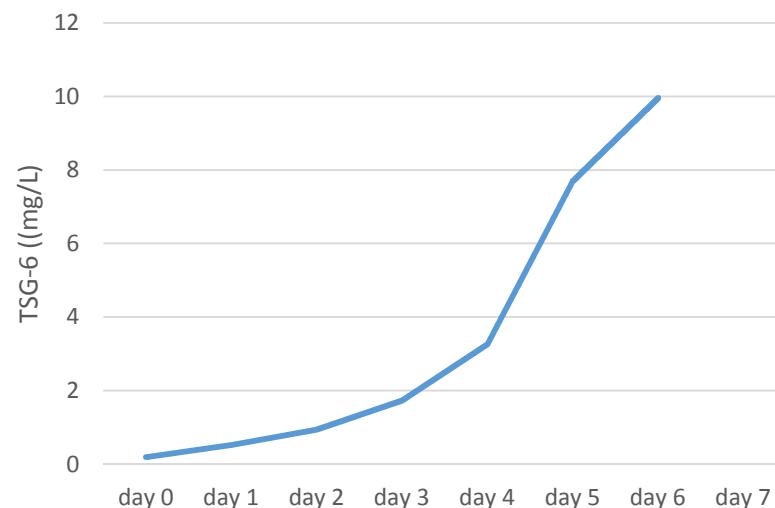
TSG-6 (Bio Reactor\_1)



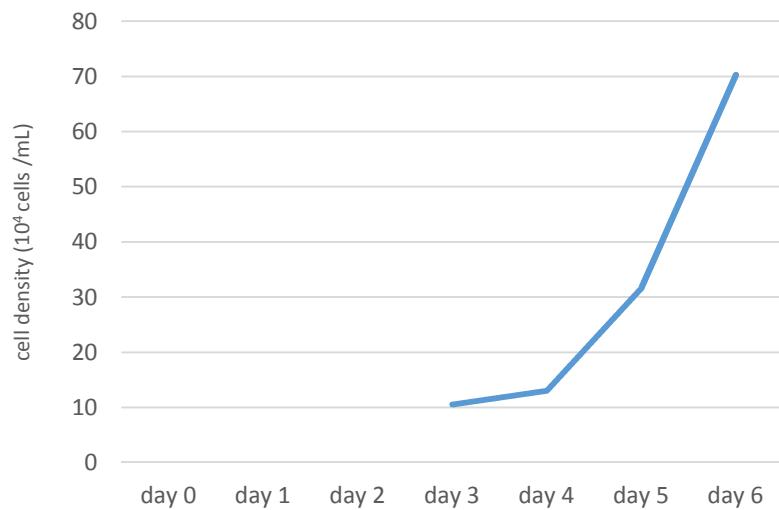
TSG-6 (Spinner\_2)



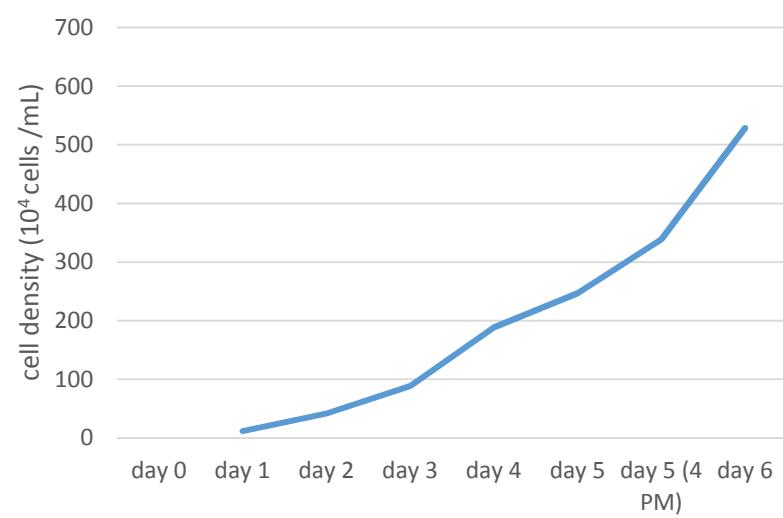
TSG-6 (BioReactor\_2)



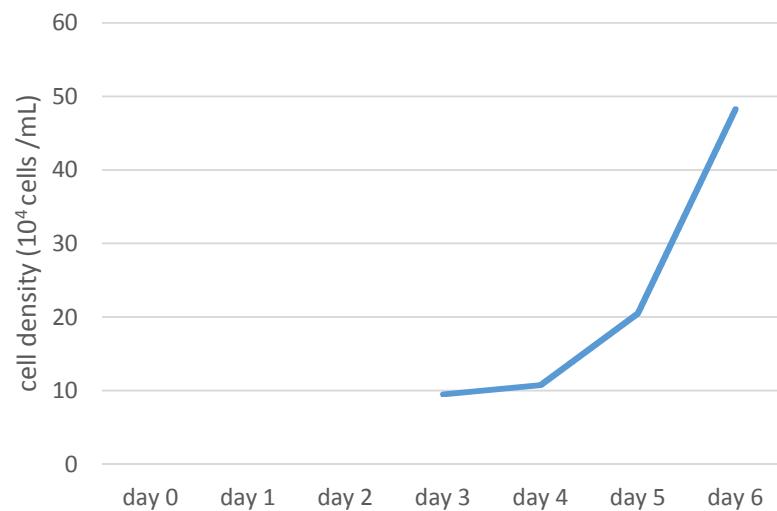
Spinner\_3



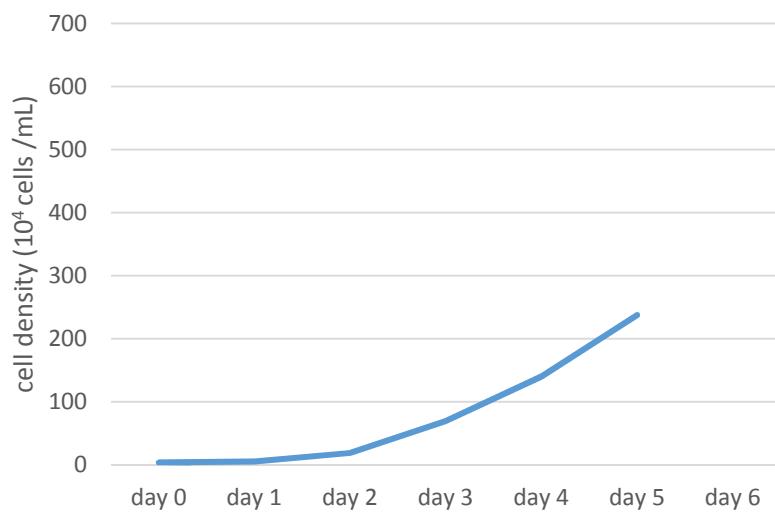
Bio Reactor\_3

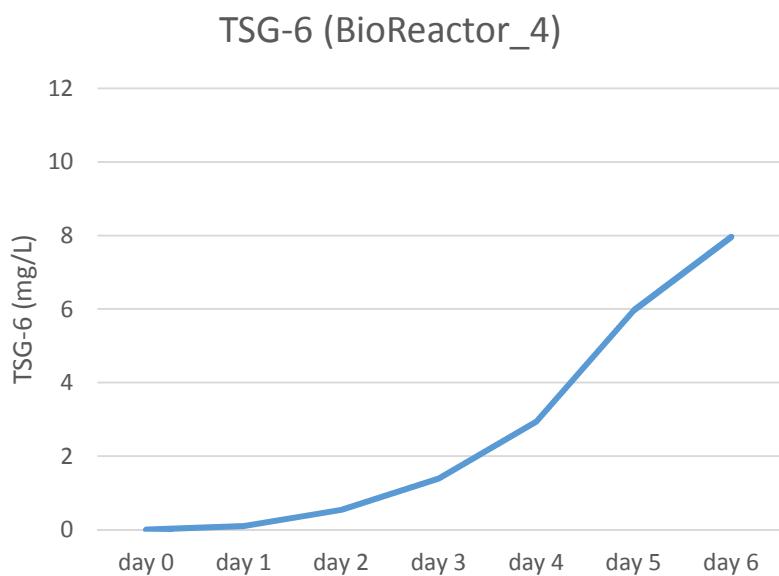
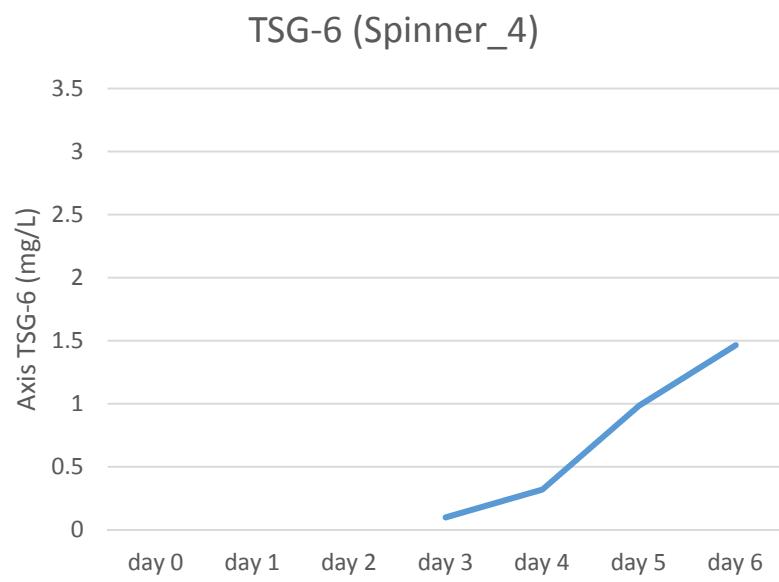
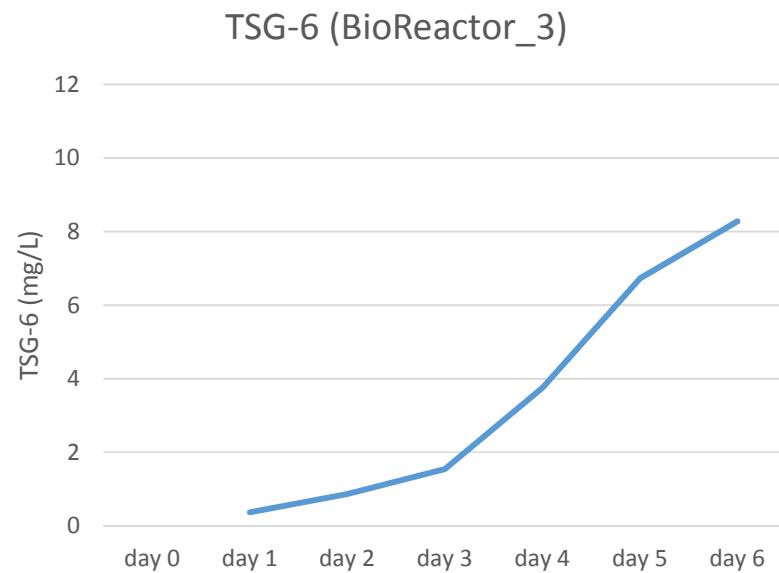
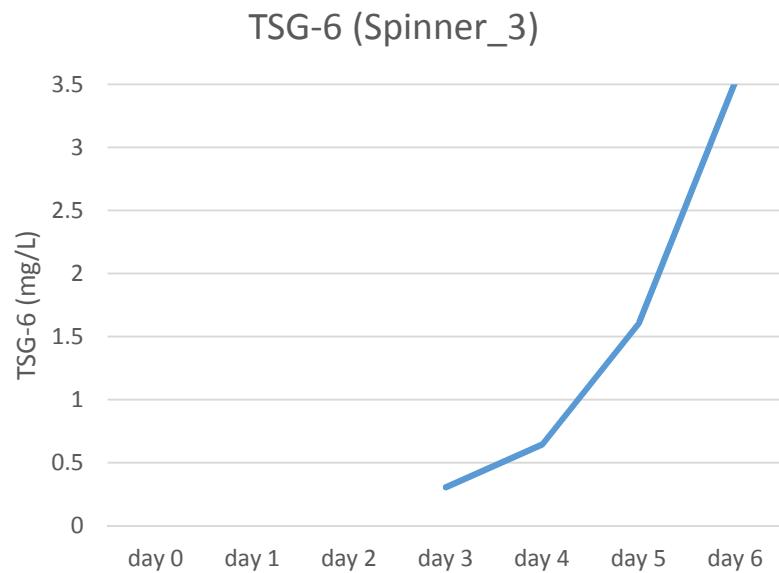


Spinner\_4



Bio reactor\_4





## Appendix 4

# Comprehensive Profiling of Alkali Injuries to the Cornea

Hosoon Choi<sup>1,2</sup>, Casie Phillips<sup>1,2</sup>, Darwin Prockop<sup>2</sup>, Joo Youn Oh<sup>4</sup>, Roxanne Reger<sup>2</sup>, Eileen Stock<sup>3</sup>, Samuel Fulcher<sup>1</sup>

<sup>1</sup>Central Texas Veterans Research Foundation Temple, TX

<sup>2</sup>Institute for Regenerative Medicine, Texas A&M Health Science Center College of Medicine at Scott & White, Temple, TX

<sup>3</sup>Baylor Scott and White Health Care Temple, TX

<sup>4</sup>Department of Ophthalmology, Seoul National University Hospital, Seoul, Korea

## **Objective/Purpose;**

Corneal chemical injuries may cause extensive tissue damage which can result in permanent visual impairment. These injuries often result from accidents occurring in the home or work place, however no effective therapy exists for severe chemical injuries of the cornea. Numerous cellular interactions and alterations occur after corneal chemical injury which are mediated by leukocytes, fibroblasts and endothelial cells, and are influenced by the combined actions of proteinases, growth factors, and cytokines which are directed to corneal regeneration and healing. This study comprehensively examined the effect of alkali injury to the cornea over time in order to further research in the development of a novel therapy with the anti-inflammatory protein TNF-stimulating gene 6 (TSG-6). TSG-6 may modulate the excessive inflammatory response that exacerbates the injury to the cornea caused by chemical exposure to the eye.

**Keywords;** cornea, alkali injury, rat, inflammation, chemical burn

## **Materials and Methods;**

A corneal alkali injury was produced with a 3-mm diameter circular piece of filter paper soaked in 1 N NaOH, and applied to the central cornea on the right eye for 30 seconds. Immediately after alkali exposure, the ocular surface was rinsed with 50 mL of PBS for 2 minutes. Gross examination for cornea opacity was performed under a dissecting microscope. The injured corneas were fixed in 10% buffered formalin and embedded in paraffin. Tissues were stained with hematoxylin and eosin (H&E) for histopathological examination, and were examined with immunohistochemistry. Neutrophil infiltration was examined by assays for myeloperoxidase (MPO) which is contained within neutrophil granules. Real-time PCR (RT-PCR) was used to evaluate mRNA expression levels of cytokines, chemokines, and genes involved in neovascularization, lymphangiogenesis and fibrosis.

## **Results and Conclusion**

Corneal opacity rapidly developed after injury by day 1, and persisted throughout the study period (21 days). Corneal neovascularization developed as early as day 1, and increased over the entire study period. The inflammatory response as measured by biochemical markers correlated with concentration of NaOH applied and began within 2 hours of injury, and persisted throughout the study period. Neovascularization, lymphangiogenesis and fibrosis progressed throughout the time course of the study period.

**Presentation Type;** Free papers **Topic Categories;** Cornea, External Eye Diseases, Eye Trauma

# Appendix 5



# Inflammation Modulatory Protein TSG-6 for Chemical Injuries to the Cornea

MR130174

W81XWH-14-1-0495

PI: Samuel F.A. Fulcher MD

Org: Central Texas Veterans Foundation Award Amount: \$985,149

## Study/Product Aim(s)

- **Aim 1.** Determine the timing and patterns of inflammation and other cellular and molecular changes in response to the severity of alkali injury. The data will allow us to select the optimal conditions for evaluating the effectiveness of TSG-6 therapy in Aims 2 and 3.
- **Aim 2.** Establish the optimal dose and the time window for effective topical and anterior chamber administration of TSG-6 therapy as a function of the severity of the alkali injury.
- **Aim 3.** Establish the optimal dose and the time window for effective intravenous administration of TSG-6 as a function of the severity of the alkali injury as well as combined topical and IV administration.

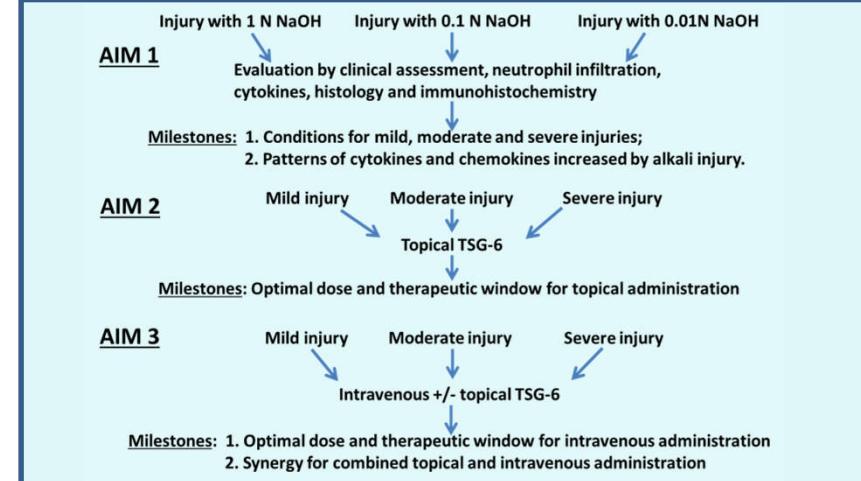
## Approach

Expose the corneas of rats to varying concentrations of alkali. Assays of inflammatory markers and clinical grading of injury and healing will be used to assess effectiveness of treatment. The results will establish the limits under which the limbal epithelial stem cells can still be rescued by modulating inflammation with TSG-6.

## Timeline and Cost

Activities	CY	14	15	16	17
Aim 1. Establish appropriate conditions for testing TSG-6.					
Aim 2. Optimal dose and time window for topical, AC TSG-6.					
Aim 3. Optimal dose and time window for intravenous and topical + IV TSG-6.					
<b>Estimated Budget (\$K)</b>	<b>\$298</b>	<b>\$356</b>	<b>\$331</b>	<b>\$985</b>	

Updated: (10/1/2015)



## Outline of Proposed Project

### Goals/Milestones (Example)

**CY14 Goal** – Establish the appropriate conditions for testing TSG-6

Determine timing and patterns of cellular and cytokine inflammatory responses as a function of alkali injury severity.

**CY15 Goals** – Optimize treatment parameters for topical TSG-6.

Optimize topical dose of TSG-6  
 Determine time window for topical therapy

**CY16 Goal** – Optimize treatment parameters for intravenous TSG-6.

Optimize intravenous dose of TSG-6  
 Determine time window for intravenous therapy  
 Determine synergistic effects of combined topical and intravenous TSG-6.

### Comments/Challenges/Issues/Concerns

- Topical and AC therapy are ineffective, and the time window for treatment is not possible to determine, Aim 3 will begin this year.

### Budget Expenditure to Date

Projected Expenditure: \$298,231

Actual Expenditure: \$262,492 (as of 8/10/2015)